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14. ABSTRACT

This research program consisted of nine interacting projects directed by ten Principal Investigators. The overarching goal of the program was to understand how experience and learning influence olfactory functioning at the molecular, cellular, neural and behavioral levels. Four of the projects used genetically engineered mouse models to investigate basic olfactory mechanisms. Five complimentary projects focused on olfactory processing in humans. Techniques employed included sophisticated brain imagining in both animal models and humans, use of

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Report Title

Learning and Olfaction: Understanding and Enhancing a Critical Information Channel

ABSTRACT

This research program consisted of nine interacting projects directed by ten Principal Investigators. The overarching goal of the program was to understand how experience and learning influence olfactory functioning at the molecular, cellular, neural and behavioral levels. Four of the projects used genetically engineered mouse models to investigate basic olfactory mechanisms. Five complimentary projects focused on olfactory processing in humans. Techniques employed included sophisticated brain imagining in both animal models and humans, use of state-of-the-art psychophysiological measures of arousal and emotion, and a variety of behavioral paradigms. Results, described in detail in individual reports, included new insights into physiological, neural and psychological mechanisms underlying olfactory learning and the role of learning and stress in human odor production and response. These results have relevance to both basic understanding of olfaction and to practical issues such as those related to ameliorating post traumatic stress disorder, olfactory loss due to traumatic brain injury or exposure to toxic volatile chemicals. The program also included a seminar program focused on the common interests of the ARO-supported research team and a one day symposium on olfaction and learning that brought 7 outstanding outside investigators to Monell to interact with Monell PIs, staff and ARO representatives.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
01/24/2013	22.00	Stacie S. Miller, Amy R. Gordon, Mats J. Olsson, Johan N. Lundstrom, Pamela Dalton. Mind Over Age Stereotype Activation and Olfactory Function, Chemical Senses, (11 2012): 167. doi:
01/24/2013	25.00	Janina Seubert, Jessica Freiherr, Jelena Djordjevic, Johan N. Lundström. Statistical localization of human olfactory cortex, NeuroImage, (02 2013): 333. doi: 10.1016/j.neuroimage.2012.10.030
04/25/2012	13.00	S. M. Khamis, R. A. Jones, A. T. C. Johnson, G. Preti, J. Kwak, A. Gelperin. DNA-decorated carbon nanotube-based FETs as ultrasensitive chemical sensors: Discrimination of homologues, structural isomers, and optical isomers, AIP Advances, (06 2012): 22110. doi: 10.1063/1.4705394
05/08/2012	14.00	Johannes Reisert, Alan Gelperin. When does more give less in the olfactory system?, Physiology News, (04 2012): 18. doi:
07/20/2012	15.00	Jae Kwak, Claude C. Grigsby, Mateen M. Rizki, George Preti, Mustafa Köksal, Jesusa Josue, Kunio Yamazaki, Gary K. Beauchamp. Differential binding between volatile ligands and major urinary proteins due to genetic variation in mice, Physiology and Behavior, (08 2012): 112. doi:
08/14/2012	19.00	J. Seubert, J. Freiherr, J. Frasnelli, T. Hummel, J. N. Lundstrom. Orbitofrontal Cortex and Olfactory Bulb Volume Predict Distinct Aspects of Olfactory Performance in Healthy Subjects, Cerebral Cortex, (08 2012): 0. doi: 10.1093/cercor/bhs230
12/03/2012	21.00	Jae Kwak. Challenges in quantitative analyses for volatile organic compounds bound to lipocalins, Journal of Separation Science, (11 2012): 2929. doi: 10.1002/jssc.201200438
TOTAL:	**7**	

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received		Paper
01/24/2013	23.00	Fredrik Åhs, Stacie S. Miller, Amy R. Gordon, Johan N Lundstrom. Aversive Learning increases sensory detection sensitivity, Biological Psychology, (02 2013): 135. doi:
TOTAL:		**1**

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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TOTAL:		

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

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TOTAL:		

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01/24/2013	24.00	Jae Kwak, Claude C. Grigsby, George Preti, Mateen M. Rizki, Kunio Yamazaki, Gary K. Beauchamp. Changes in volatile compounds of mouse urine as it ages:their interactions with water and urinary proteins, Manuscript in preparation for submission to a journal (10 2012)
02/22/2012	2.00	Stacie S. Miller, Amy R. Gordon, Mats J. Olsson, Johan N. Lundstrom, Pamela Dalton. Mind over age - Social priming and olfactory function, ( )
02/22/2012	6.00	Fredrik Åhs, Amy R. Gordon, Stacie S. Miller, Johan N. Lundström. Aversive learning increases sensory detection sensitivity, ( )
02/22/2012	1.00	Julie A. Mennella, Allison Steinmeyer, Jae Kwak, Malek Kamoun, Maryanne Curran Opiekun, Kunio Yamazaki, Gary K. Beauchamp. Human Odortypes: Stability and HLA Dependence, ( )
02/22/2012	5.00	Cristina Jaén , Pamela Dalton . White Paper: Learning, Odors and Stress in Asthma, ( )
02/22/2012	4.00	Johannes Reisert, Alan Gelperin. When does more give less in the olfactory system?, ( )
02/22/2012	3.00	Pamela Dalton. There's something in the air: Effects Of Beliefs And Expectations On Response To Environmental Odors, ( )
02/23/2012	10.00	Jae Kwak, George Preti. CHALLENGES IN THE INVESTIGATION OF VOLATILE DISEASE BIOMARKERS IN URINE, ( )
02/23/2012	8.00	JAE KWAK, CLAUDE C. GRIGSBY, MATEEN M. RIZKI, GEORGE PRETI, JESUSA JOSUE, KUNIO YAMAZAKI, GARY K. BEAUCHAMP. Differential Binding between Volatile Ligands and Major Urinary Proteins Due to Genetic Variation in Mice, ( )
02/23/2012	9.00	Johan N. Lundström , Beverly J. Cowart . An approach to establish non-invasive measures from the human olfactory bulb, ( )
03/01/2012	11.00	Johannes Reisert, Koichi Matsumura, Alan Gelperin. Dynamics of Active Odor Sampling during Odor-Guided Decision Making in the Mouse, Manuscript in preparation for submission to a journal (03 2012)
03/05/2012	12.00	Samuel Khamis, Ryan Jones, A.T. Charlei Johnson, George Preti, Jae Kwak, Alan Gelperin. DNA-decorated carbon nanotube-based FETs as ultrasensitive chemical sensors: Discrimination of homologues, structural isomers, and optical isomers, Manuscript in preparation for submission to a journal (03 2012)

03/27/2013 26.00 Nicholas J. Kybert, Mitchell B. Lerner, Jeremy S. Yodh, George Preti, A.T. Charlie Johnson. Differentiation of Complex Vapor Mixtures Using Versatile DNA-Carbon Nanotube Chemical Sensor Arrays ,
Submitted (02 2013)

03/27/2013 27.00 Katharine A. Prokop-Prigge , Erica Thaler , George Preti . Identification of volatile organic compounds in human earwax,
Submitted for publication (02 2013)

07/31/2012 16.00 Pamela Dalton, Richard Doty, Claire Murphy, Howard Hoffman, Christopher Maute, Jerry Slotkin. Olfaction assessment using the NIH toolbox,
Submitted for publication (07 2012)

07/31/2012 17.00 Jae Kwak. Challenges in quantitative analyses for volatile organic compounds bound to lipocalins,
Submitted for publication (06 2012)

07/31/2012 18.00 Karin B. Jensen , Jessica Freiherr , Johannes Frasnelli , Johan N. Lundström . Pain processing in the human brain: An ALE meta-analysis of neuroimaging studies in patients and healthy controls,
Submitted for publication (07 2012)

TOTAL: **17**

Number of Manuscripts:

Books

Received Paper

08/15/2012 20.00 Pamela Dalton. Olfactory Cognition, Amsterdam / Philadelphia: John Benjamins , (01 2012)

TOTAL: **1**

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Enjieu Kadji, Herve Germain	1.00
Golden, Glen J.	1.00
Ham, Hyoung-Geol	0.85
Jaen, Cristina	1.00
Kwak, Jae H	0.33
Pelchat, Marcia L.	0.10
Seubert, Janina	0.53
FTE Equivalent:	**4.81**
Total Number:	**7**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Beauchamp, Gary K	0.16	
Cowart, Beverly J	0.45	
Dalton, Pamela H.	0.30	
Gelperin, Alan	0.31	
Lowe, Graeme	0.64	
Lundstrom, Johan N	0.47	
Preti, George	0.27	
Reisert, Johannes	0.29	
Wysocki, Charles J	0.56	
Yamazaki, Kunio	0.53	
FTE Equivalent:	**3.98**	
Total Number:	**10**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Mayro, Eileen	0.02	Biopsych
Tadas, Hardshad	0.18	Computer Science
Zeb, Salma	0.04	Psychology
FTE Equivalent:	**0.24**	
Total Number:	**3**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
Chai, Jinghua	0.20
Eades, Jason	0.31
Gregory, Kristen	1.00
Kathleen Mueller	0.37
Maute, Christopher	0.81
Milbury, Lydia	1.00
Opiekun, Maryanne	1.00
Varallo, Lauren	1.00
Weiss, Brian	0.67
Wilson, Tamika	0.55
FTE Equivalent:	**6.91**
Total Number:	**10**

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

Preface

Alan Gelperin and Gary Beauchamp

This research program, “Learning and Olfaction: Understanding and Enhancing a Critical Information Channel” consisted of nine separate research Projects headed by ten Principal Investigators. Here we provide a brief overview on the interacting themes of the program.

Four of the projects used animal models and five involved studies of human subjects, but all focused on a central theme: mechanisms and functions of olfactory learning. Olfaction, one of the most primordial senses, evolved to provide the organism with critical information about its chemical environment – both to protect against danger (e.g., from predators, diseases, or poisons) and to inform it of the presence of nutrients and other members of its species (e.g. for identifying food or for mate choice or offspring care). Thus profound problems of survival and reproductive success depend on olfaction, and this is mirrored in the fact that some of the oldest parts of the vertebrate brain, those that in humans process emotion most intensely, are directly connected to, and even arose out of, the olfactory system. Thus a second theme evident in many of the projects was the role of emotion and stress in odor learning, odor memory, and even odor production.

Projects 1, 2, 4 and 7 were designed to investigate basic mechanisms of odor learning in the CNS. The first three of these sought to identify neural pathways that were modified by exposures to individual odorants in various mouse model systems. Project 7 used brain imagining techniques to evaluate CNS processing following odor learning in humans. Single odorants provide superb control for many experimental studies, but in the real world mixtures of odorants are the rule and thus these Projects spanned the gamut of odor complexity: single odorants (Projects 1, 2, 4, and 9); complex odorant mixtures (Projects 6 and 7); and body odors of social significance (Projects 3, 5, and 8). Also, odor exposure paradigms vary across these Projects. Some involved passive exposure to odorants (e.g. Projects 2, 4, and 5), whereas others employed active training techniques to study odor learning (e.g., Projects 3, 6, 7, and 9). Together the results of these different ways of studying odor learning have provided a rich source of information about how odors are processed and incorporated into everyday life.

As indicated above, the effects of stress and emotion on odor processing played a central role in a number of the Projects. Projects 7, 8 and 9 directly induced stress to evaluate how this affected odor learning and memory. Project 5 turned the table to ask how specific odor sources (odorant mixtures compared to body odors from siblings or strangers) could activate or reduce stress. Thus all of these Projects engaged the

important issue of how odors modulated emotion, and in turn, how emotion could modulate responses to odors.

State-of-the-art techniques and psychophysical methods characterized all nine Projects. Genetically engineered mouse models provided powerful tools to investigate basic issues of learning in Projects 1 – 4. Along with these mouse models, these Projects also employed sophisticated analytical approaches to data gathering and analysis. For example, methods to monitor sniffing in real time (Project 1), methods to chemically ablate olfactory functioning (Project 2), and methods to scan multiple layers of olfactory bulb slices (Project 4) were employed. In the human studies (Projects 5 – 9) equally novel and powerful techniques were used. For example, in Project 6, a potentially breakthrough technique for monitoring olfactory bulb activity in humans was developed. This Project also employed sophisticated CNS monitoring as did Project 7. A significant strength of a number of the human Projects (5, 8, and 9) was the use of a variety of psychophysiological measures of stress as dependent measures.

These projects were, by design, basic in nature. Yet they had many potential implications for the military as well as the general public. An understanding of how stress and odor interact has direct implications for issues of post-traumatic stress disorder as odor has been identified as an important trigger for symptoms of this syndrome. This makes sense based on the direct functional connection between olfaction and emotion. Principles learned in several of these Projects (e.g., 5, 7, and 9) may provide practical insights into treatment options. Project 4 investigated the role of nitric oxide (NO) in odor learning at a very basic level. But if this gas is importantly involved in odor learning, one might speculate that various common drugs that humans, including warfighters, consume and that alter NO metabolism may also affect olfactory processing and olfactory learning. Projects 3, 5, and 8 explored the complexities of body odors. These odors, which evolved to communicate messages between individual organisms, could potentially be decoded to obtain information relevant to a wide variety of military and diagnostic endpoints.

These Projects were, by design, extremely diverse in methods, techniques, approaches, and animal model systems. For those reasons, we thought that it would be most appropriate to explain in detail the accomplishments of each project independently during the 2 year funding period.

Each project report includes a statement of the problem studied and a summary of the most important results achieved. Each report also contains the pertinent illustrations and tables needed to support those results.

Table of Contents

Project 1: Effects of olfactory marker protein on patterns of olfactory sampling

Alan Gelperin and Glen Golden

Executive Summary

We developed a unique method using implanted telemetry sensors to stably record respiration over long time periods in freely moving mice performing an olfactory discrimination task. In order to accomplish this, we modified/improved a commercially available olfactometer that offered the odor stimulus and the water reward in a single port to separate odor sampling and water reward collection in separate ports, allowing us to accurately record odor sampling time.

We developed odor discrimination protocols to test the behavioral responses of wild-type and genetically modified mice to various concentrations of an odorant using an ascending and descending method of limits test. We also developed odor discrimination protocols to test the behavioral responses of mice to various concentrations of an odorant following prolonged exposure to a familiar and novel odorant.

We validated the method of thoracic pressure sensor telemetry. This was accomplished by implanting nasal cannulas in mice already implanted with thoracic telemetry sensors and comparing the respiration signals from the thoracic sensors and a sensor in the nasal cannula. We created custom software to pair behavioral odor discrimination data with respiration data derived from implanted thoracic telemetry sensors.

In summary, by using this unique olfactometry and telemetry system, we characterized sniffing strategies used by olfactory marker protein (OMP) knock-out and background strain wild-type mice in olfactory discrimination tasks, in order to clarify the role of OMP in olfactory sensory neuron function.

Background and Objectives

The goal of these experiments is to determine the normal patterns of active odorant sampling and how patterns of odorant sampling vary both with the cognitive demands of the odorant sampling task and with the history of prior odorant exposure. We measure odorant sampling patterns in both wild type mice and in mice in which olfactory marker protein (OMP), a protein important for olfactory transduction, has been genetically deleted. Mice lacking OMP display a slowed electro-olfactogram response to odorant stimulation[1] and reduced odorant sensitivity and altered odorant quality perception when tested behaviorally[2,3]. The details of how OMP functions in olfactory transduction remain a mystery. We are preparing to perform similar experiments measuring both olfactory recognition and decision making behaviorally while measuring breathing and sniffing rates with an implanted wireless sensor using additional strains of mice in which other proteins considered important in olfactory transduction have been eliminated (i.e., NCKX4 – a potassium-dependent Na+/Ca2+ exchanger and ANO2 – a calcium-activated chloride channel) in comparison to wild type mice.

Behavioral experiments in rodents and humans show that a single odorant sample or sniff approximately 250 ms in duration can be sufficient to reliably distinguish between two odorants. Given a complex discrimination task, mice and humans can improve their odorant discrimination accuracy by temporal integration of the stimulus, i.e., by taking more or longer odorant samples before making a decision they can trade off speed of discrimination for accuracy of discrimination[4]. Understanding the temporal framework in both the frequency of

sniffing and the duration of odor sampling in wild type animals and in OMP knockouts and other genetically engineered mouse strains will help to elucidate the role of proteins reported to be involved in olfactory transduction. The results of this behavioral work will be compared to biophysical data on odorant responses of individual olfactory receptor neurons isolated from both wild type and genetically engineered mice.

Production of OMP knockout mice and wild type mice

We are currently generating a sustained line of OMP F2 knockout (KO) and wild type (WT) mice. We have completed production of the first generation of hybrids (F1) between OMP KO mice and background wild type mice. The F1 mice with OMP +/- genotype have been mated to mice also carrying the OMP +/- genotype to produce an F2 generation. Approximately 25% of the F2 mice were expected to be OMP +/+, 25% were expected to be OMP -/-, and 50% were expected to be the OMP +/- genotype. The actual distribution resulting form mating OMP+/-to OMP+/- mice revealed a higher number of OMP-/- knockout mice in comparison to the number of WT mice. The OMP +/+ and the OMP -/- are being used for odor discrimination and adaptation experiments (see below). We will continue breeding these mice to expand the numbers of mice with genotypes suitable for measurements of the biophysics of their olfactory receptors. We are also in the planning stages for breeding NCKX4 and ANO2 strains.

Hardware and software modifications

Three commercial olfactometers have been modified to optimize their configuration for training mice to associate odorant stimuli with water reward. The original combined odor-water port configuration in the commercial olfactometer sold by Knosys (http://knosysknosys.com/) has been replaced with separate odor and water ports. This modified configuration allows us to measure how long the mouse samples an odor prior to making a decision about its identity in the go/no go (GNG) discrimination task. In the GNG task the mouse uses odorant identity to determine if the water port will or will not deliver a water reward. In addition, two of the olfactometers are equipped with a commercial telemetry system (Data Sciences International, St. Paul, MN, USA) in order to record wireless signals from implanted telemetric breathing sensors. The implanted breathing sensors allow the continuous measurement of respiration patterns while the mouse samples odors and makes decisions based on that odor sampling in the olfactometer. These measurements will clarify how the inactivation of OMP and previous odor experience affect odorant-based decision making, particularly speed-accuracy tradeoff. Custom data analysis software written in Matlab is currently being used to pair the sniffing and breathing pattern data with behavioral performance data taken simultaneously during the odor discrimination task so that the two data streams can be compared in a common timeframe. The software is now being tested with both behavioral and breathing pattern data generated with OMP KO and WT mice recently implanted with breathing sensors.

Experimental stage

Mice have been trained to collect a water reward when presented with the odor of 10^{-4} % saturated vapor of 1-Propanol (S+) and to withhold responding to the water port when presented with the odor of the solvent used to dilute the 1-Propanol (i.e., mineral oil [MO]) serving as the S- in the GNG odor discrimination task. 1-Propanol is a commonly used odor stimulus in olfactory discrimination tasks using OMP and other genetically engineered mice.

Following the completion of training, we implanted a dummy breathing sensor in one of the

trained mice and determined that the mouse was still able to complete the GNG discrimination task at the level of performance accuracy shown immediately prior to the implantation surgery. These results are in agreement with our previous experience that only minor perturbations in performance result in mice implanted with the wireless breathing sensors, although in previous work the breathing/sniffing sensors were implanted by an outside vendor.

We have designed tests for olfactory discrimination using both the ascending and descending method of limits. For both methods, we perform concordance (>85% or better) blocks of twenty trials (10 S+; 10 S-) at the start and finish of each testing session to demonstrate the capability of an individual mouse to perform the required task prior to and immediately after testing. We also employ a block of MO vs. MO to ensure that the mice are using odor stimuli and not other cues to make their choices. The ascending method of limits uses two unrewarded trial probes of a test concentration included in a block of 9 S+ and 9 trials. We increase the test concentration by a half log step each session until the mice are reliably reporting to the water reward port even though no reward is available. The descending method of limits replaces the S+ stimulus (5 rewarded test stimulus trials; 5 unrewarded test stimulus trials; 10 S-) with a half log step lower concentration for each session until the mice are performing at chance. During adaptation experiments, we test the ability of the mice to perform the GNG odor discrimination task (S+ vs S-) while we fill the operant chamber with various concentrations of either the S+ stimulus, an odor stimulus that does not stimulate the same odor receptor neurons (i.e., cineole), or with the odor of MO.

Focus of activity during final six months of the grant

Eight mice (F2 generation; 4 WT; 4 OMP KO) have been implanted with pleural pressure sensors. These mice are currently being trained and/or tested in the GNG odor discrimination task. We have utilized a version of both the ascending and descending method of limits to test olfactory acuity during olfactory discrimination tasks in 3 of the implanted mice (2 KO and 1 WT). Behavioral and breathing pattern data for these mice are currently being used to test and perfect the accuracy of our custom software. The remaining mice are currently finishing their training and will begin testing shortly. We anticipate implanting sensors in several more trained OMP KO and WT mice to provide additional data.

Project 2: Experience-Dependent Changes in Responses of Olfactory Receptor Neurons

Johannes Reisert

Executive Summary

Olfactory receptor neurons (ORNs) reside in the nasal cavity and are therefore continuously exposed to the harsh outside environment, which can lead to repeated damage. This damage is mitigated because ORNs retain the ability to regenerate throughout live. We addressed the question if the ability of ORNs to regenerate can be altered by odorant exposure. We found that indeed, ORNs can regenerate in higher numbers during odorant exposure, but this effect is specific to ORNs that respond to the odorant. ORNs that are not activated remain unaffected. But, although responsive ORNs exist in higher numbers, the targeting of their axons to the olfactory bulb is less precise compared to cells that do not respond. These data demonstrate that odorant expose can have multiple effects on regenerating ORNs and that ORNs are affected in a complex manner.

Background and Objectives

Olfaction begins with the binding of an odor molecule to odorant receptors (ORs) embedded in the cilia of olfactory receptor neurons (ORNs). This activates a signal transduction cascade that culminates in the generation of action potentials that are carried to the olfactory bulb via axons. All ORNs that express one type of ~1000 different ORs in the mouse send their axons to 2 – 4 glomeruli in the bulb, where they make connections with second order neurons.

We investigated how long term odorant exposure can alter ORN physiology, psychophysical perception[5,6] and axonal targeting to glomeruli in the olfactory bulb. In other words, how plastic is the peripheral olfactory system and what is the role of odorant exposure in these processes. In particular, we are interested in these processes following damage to the olfactory epithelium. We investigated the number of ORNs in the nasal cavity and if axonal targeting to the bulb has changed. For this purpose we used genetically modified mouse lines that express green fluorescent protein (GFP) in ORNs expressing a known odorant receptor. Mice were exposed to odorants known to activate or block ORs. Electrophysiological techniques to monitor the odorant-induced electrical activity of ORNs and immunohistochemical methods to investigate axonal targeting were employed.

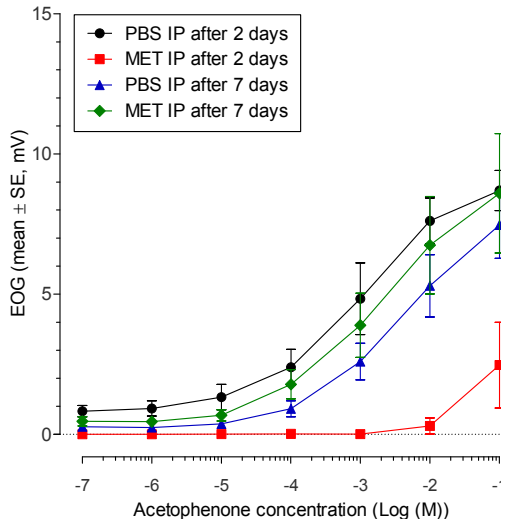


Fig. 1 Methimazole-meditated ablation greatly reduced olfactory responses. Mice were injected with PBS (control) or methimazole and EOG responses to acetophenone recorded after 2 and 7 days.

First we exposed mice for 10 days to the odorant a and recorded olfactory mucosal field potentials (electroolfactograms (EOGs)) in response to heptanal. For this exposure paradigm, no clear difference in EOG response was observed between heptanal exposed and control mice. Also, the volume of heptanal-responsive glomeruli was not changed (data not shown). This lack of effect

might arise from two factors: The exposure duration was not long enough and/or the turnover of ORNs was too slow to allow for new ORNs (either positively or negatively affected by the presence of heptanal) to be generated. We thus altered our approach by first ablating the olfactory epithelium by methimazole injection[7] and exposing mice to odorants during the re-growth of the epithelium thereafter for up to 21 days. The rationale behind this approach is that odorant exposure will have a larger effect during a regenerating epithelium rather than on an already existing one. Methimazole injection almost entirely abolished EOG responses recorded two days post injection, demonstrating that the olfactory epithelium has been ablated nearly entirely. EOG responses recovered to levels comparable to control conditions after seven days (Fig. 1).

Bulbar targeting

Bulbar targeting was investigated in genetically modified mice expressing GFP in ORNs that express the I7 odorant receptor. This receptor is activated by heptanal and inhibited by

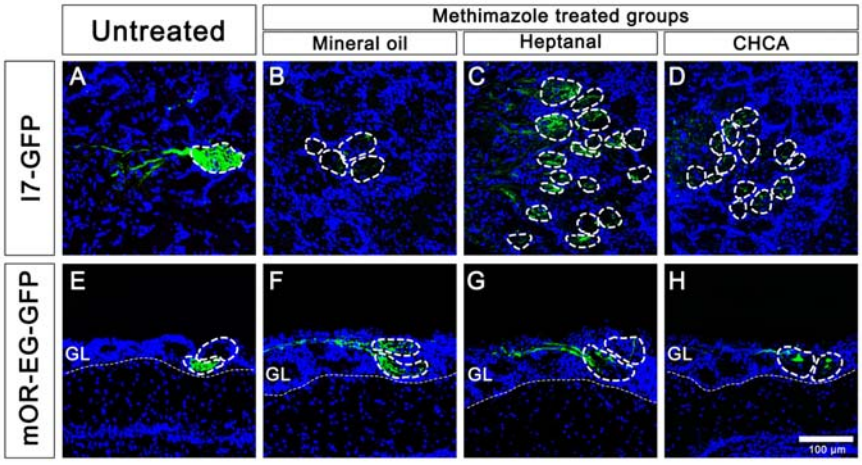


Fig. 2 ORN axon targeting to glomeruli in the olfactory bulb.
A A well-defined glomerulus in an untreated I7-GFP mouse. Following epithelial ablation mice were exposed to mineral oil (B) heptanal (C) or chca (D). Olfactory bulb sections were stained with a nuclear (DAPI in blue) stain, green is GFP fluorescence from olfactory axons of I7 ORNs. White circle outlines a single glomerulus as defined by lack of blue nuclei. E – F are bulbar sections from mice that express GFP with the mOR-EG odorant receptor and that underwent the same experimental paradigm, GL: glomerular layer.

cycloheptanecarbaldehyde (chca). In a control (untreated) mouse, axons originating from I7 ORNs converge precisely onto two glomeruli in each bulb (Fig. 2A) with very few nerve fibers innervating neighboring glomeruli.

Following methimazole treatment mice were exposed to either the carrier mineral oil as a control (B), heptanal (C) or chca (D) for 10 – 21 days to allow for the epithelium to regenerate and send axons to the olfactory bulb. 10 days proved to be insufficient to allow ORN axons to reach the bulb and no GFP fluorescence was observed. After 21 days solid axonal targeting (as judged by the appearance of newly-grown GFP labeled green nerve fibers) was observed into many glomeruli in mice that were exposed to heptanal (Fig. 2C) but significantly less innervation when exposed to mineral oil (B) or chca (D). Interestingly, in neither case, single glomeruli were formed by the innervating new neurons as is

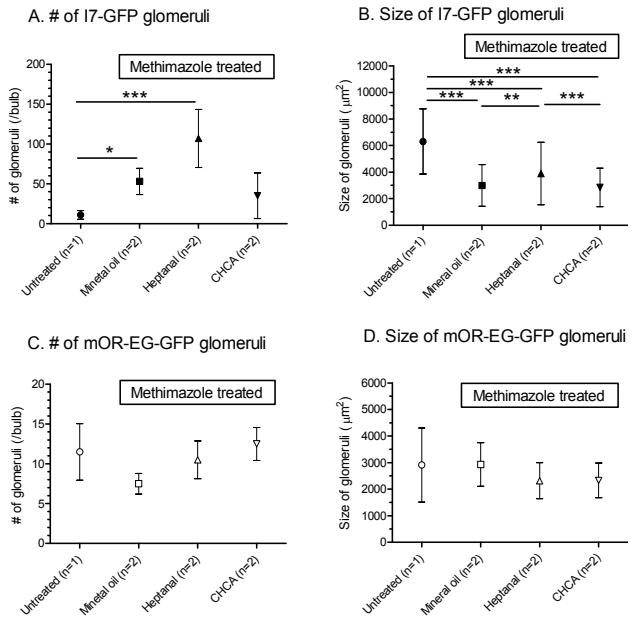


Fig. 3 Odorant exposure alters glomerular size and number of innervated glomeruli in I7-GFP mice.
A, B Distribution of glomerular size in I7-GFP (untreated), methimazole treated and mineral oil, heptanal or chca exposed mice. "n" number of mice. C, D same experiments, but for mOR-EG-GFP mice. Values are mean ± SD.

observed in mice which did not have their epithelia ablated (A). As a control, we repeated the same experiments with a different mouse line that expresses GFP with the OR mOR-EG. This OR responds to eugenol, but not heptanal or chca. In this case, regardless of exposure condition following epithelial ablation, mOR-EG expressing ORNs still converge on only 1 or 2 glomeruli.

Fig. 3 shows a quantification of the observed targeting defects. While untreated mice had only very few glomeruli in both the I7 and the mOR-EG mice, I7 mice showed a marked increase in innervated glomeruli following ablation even when exposed to mineral oil, which was further increased when the mice were exposed to the agonist heptanal, but not the antagonist chca (Fig. 3A). Glomerular size decreased for all exposure conditions following ablation (Fig. 3B). In contrast, mOR-EG mice showed very little difference in both the number of innervated glomeruli and glomerular size (Fig. 3C&D).

Olfactory receptor neuron regeneration

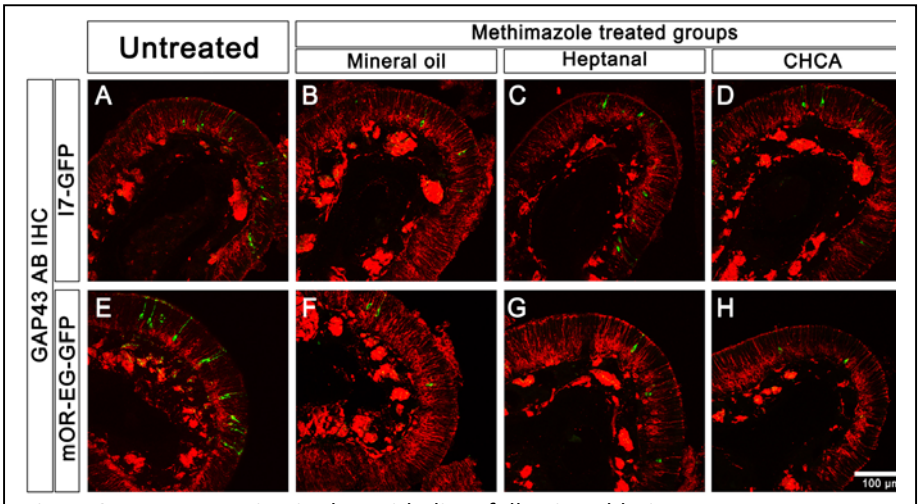


Fig. 4 ORN regeneration in the epithelium following ablation.
Epithelial sections were stained with a GAP43 antibody (red) and also investigated for GFP, expressed either in I7 (A – D) or mOR-EG (E – H) ORNs. Following epithelial ablation mice were exposed to mineral oil (B, F) heptanal (C, G) or chca (D, H).

Considering the observed changes in the olfactory bulb, we next investigated the odorant-exposure dependent recovery in the olfactory epithelium 21 days after ablation. Fig. 4 shows olfactory turbinates stained for GAP43, a marker of immature (or regenerating) ORNs. Again, GFP labeled mice are used, ORNs that either express the I7 (Fig. 4A – D) or the

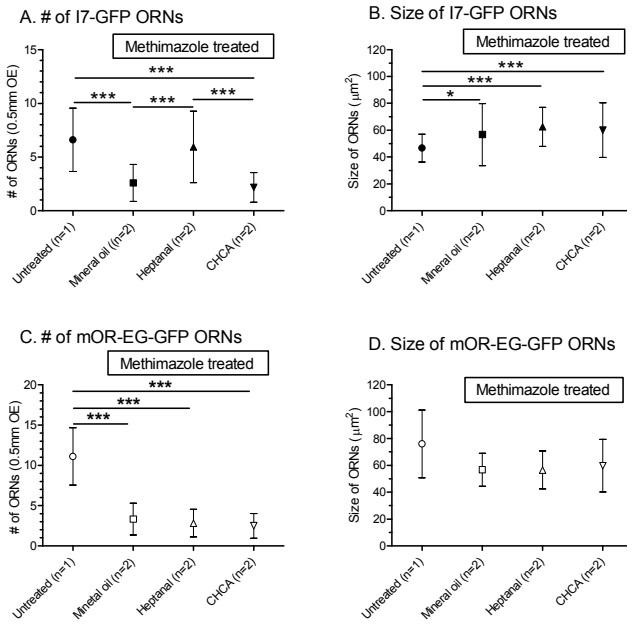


Fig. 5 Odorant exposure alters the number of ORNs expressing the I7 odorant receptor.
A, C Number of I7 or mOR-EG expressing ORNs in control (untreated), methimazole treated followed by mineral oil, heptanal or chca expose. "n" number of mice. B, D ORN cell body size remained unchanged in both I7 and mOR-EG ORNs. Values are mean ± SD.

mOR-EG (Fig. 4E – F) OR are also GFP positive. For all methimazole-treated mice, an increase in GAP43 positive cells was observed compared to untreated (un-ablated, A, E) mice as expected for a regenerating epithelium. For both I7 and mOR-GFP mice epithelial ablation greatly reduced the number of GFP positive ORNs (Fig. 4 and Fig. 5A, C) which was rescued when I7 mice were exposed to their agonist heptanal but not their antagonist chca. mOR-EG ORNs did not show a change when exposed to either heptanal or chca compared to mineral oil. The size of the ORN cell body remained the same in all cases (Fig. 5B, D).

Conclusions and future directions

The olfactory epithelium is a continuously regenerating epithelium and we posed the question if odorant exposure can alter the makeup of the epithelium over time. In particular we investigated if ORNs expressing one of two odorant receptors, either the I7 or the mOR-EG OR, were differentially altered following epithelial ablation. Indeed, exposing ORNs to their cognate ligand can increase their number in the regenerating olfactory epithelium, but it also leads to large mis-targeting to the glomeruli in the olfactory bulb. But it seems that true activation of the ORNs is required since an antagonist that will bind to the OR but does not activate it (as is the case with chca and the I7 OR), does not lead to an increase in ORN numbers in the epithelium and not to mis-targeting to the same extent. It is also specific to an odorant – odorant receptor pair (heptanal and the I7 OR), since exposure of mOR-EG ORNs to its non-ligand heptanal does not have any effect. Future work will address how ORN function depends on the odorant-exposure regime. This research can help to understand what role odorant exposure (and therefore ORN activity or lack thereof in case of an ORN inhibitor) might play during the regrowth of damaged nasal sensory tissue in the nose itself, how odorants affect the connectivity of ORNs to the olfactory bulb and how odorant exposure can alter the physiology of ORNs.

Project 3: Genetics of Olfactory Identity

Kunio Yamazaki and Gary Beauchamp

Executive Summary

The issues addressed in this work were two-fold: **(1)** We wanted to understand why it was so much more difficult to train mice to discriminate two different humans than to train them to discriminate two different mice; **(2)** we wanted to explore the role of MHC differences, both in the trained mice and the human odor donors, in identifying human HLA-determined odortypes. Concerning issue **1**, we conclude that there were two factors underlying the different rates of learning to discriminate individual mouse odors compared with individual human odors. First, older mice, which were used in the preliminary work, learn more slowly that younger mice used in our mouse odor training. However, this probably was not a major factor. Instead similarities in the HLA types of the two donors most likely accounts for the difficulty in training in the pilot work. Concerning issue **2**, we provide additional strong evidence that individual human odor is controlled in part by variation in HLA genes and that variation in MHC types most likely provide the major source of genetic individual odor variability in humans as it does in mice. Moreover, we provide provisional evidence, which requires follow-up studies for verification, that transgenic mice that carry a human allele produce odors that share commonalities with humans with the same allele. This supports the view that a subset of individual-based odorants are common to mice and to humans. A major future goal is to identify these odorants.

Background and Objectives

Individual humans can be identified by differences in body odor as indicated by the long history of using trained dogs to follow and recognize individuals. It was generally assumed that this individual-specific body odor was due to genetic influences on body metabolites that are expelled into the air environment. However no specific genetic loci were identified until Lewis Thomas[8] speculated that genes in the major histocompatibility complex (MHC: the most variable of all genes and those that specify cell-surface recognition proteins) might fulfill this role.

The first strong experimental indication that MHC genetic variation may provision an animal with a unique body odor (its MHC odortype) came from mating preference studies that indicated that mate choice was dependent in part on MHC types of mice[9, 10]. Based mainly on the presumption that mate choice in mice is likely to be strongly influenced by odors, these investigators next conclusively demonstrated that mice of different MHC types have different odors that other mice, rats and even humans can be trained to recognize[11-13]. Subsequent studies in several laboratories have substantiated these conclusions for mice and rats, and have demonstrated a role for MHC odortypes in rodent social and sexual behavior [14-17].

These animal studies raised the obvious question as to whether humans also express unique MHC-regulated odors that could be involved in mate choice, often with the hypothesis that they would provide a mechanism for enhancing MHC diversity, insuring optimal MHC identity in offspring and avoiding inbreeding in general (reviewed in[15]). As with mice, the first suggestions that this may be the case came from studies on mate choice conducted by Ober and colleagues[18]. They demonstrated that mate choices (marriages) in Hutterites, a North American population that is reproductively isolated, is correlated with HLA type. Subsequently, some studies have suggested that mate preferences among

humans are related to MHC variation whereas other studies have not found this to be the case (reviewed in Havlicek and Roberts[19]).

There is evidence that genetic variation in the human MHC (called Human Leukocyte Antigen [HLA]) influences body odor but it is less extensive than is the case for rodents. In human studies, as with studies with other animals, there are two general approaches to determining the existence of distinct odors that are associated with variations in MHC genes. First, one can examine preferential ("natural") responses to odors collected from individuals varying in MHC type. Presuming studies are appropriately designed, if there are statistically reliable differences in preference, this implies that there must be distinct odors. In a series of studies on human body odors collected on T-shirts[20, 21], have reported data that are consistent with the hypothesis that variation in HLA types is related to variation in body odors. More recently, studies of human preferences for perfumes to which MHC peptides have been added[22] have provided additional evidence that MHC types in humans can be communicated through changes in "odor."

More direct evidence for the existence of human HLA-determined odortypes, as well as paradigms for examining their chemical nature, comes from studies that specifically examine the ability of the olfactory system to detect and discriminate odor from individual humans of differing HLA types. In pioneering studies, Ferstl and colleagues[23-25] reported that rats could be trained to discriminate between urine odors (and in one case sweat odors) of individuals of differing HLA types.

We decided a number of years ago to investigate the possible existence of HLA-determined body odors expressed in human urine using the mouse as an odor sensor. The use of the trained mouse has several advantages over the dog. First and foremost it allows control over the learning situation that is generally not present in canine studies. Second, it is considerably more cost efficient and more animals can be used efficiently. Third, as a direct consequence of these first 2 advantages, it will allow us to move more quickly identifying significant odorants and to develop devices for detection of individual odor.

Pilot studies

Training mice to discriminate urine odors of two humans. Our first task in pilot studies was to train mice in our standard Y-maze to discriminate two humans based on their urine odors. In pilot studies on which this research project was based, this turned out to be extremely difficult which was surprising since mice are easily trained to discriminate body odors of individual mice and rats are equally adept at this task. An example is provided below where a single mouse that had never been trained in the Y-maze before (see Yamaguchi et al.[13] for full description of the apparatus and procedure) is being trained to discriminate between urine samples collected on multiple days from two individual humans. As indicated, it was quite difficult to train mice to discriminate even between two individual humans (Fig 1). At the time these studies were ongoing we had not done HLA typing and thus were unaware that the HLA types of these two individuals were very similar (see Fig 1 caption).

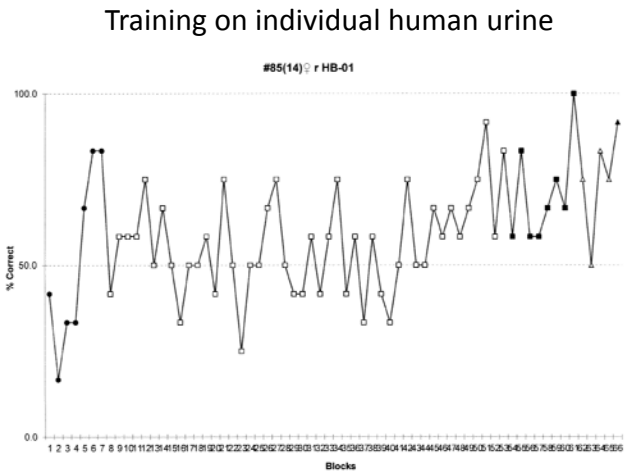


Fig 1. Training record for a single mouse to discriminate odors of human urine samples from 2 individuals with very similar HLA types (A2A3; B44B44 and A2A3; B7B27) collected over several days. Each dot represents the % correct (concordant with training) in each 12 trial blocks. Random responding is 50%. Evidence of successful learning is not apparent until after more than 700 training trials which is many more than is typically necessary to train mice to discriminate between mice differing in MHC type (see below).

Training mice to discriminate between pure odorants. In another project we trained mice in our Y-maze to discriminate between pure odorants. One group of naive mice (top, Figure 2) was trained to discriminate between two odorants that humans can easily distinguish by smell whereas the second group of mice (bottom, Figure 2) was first trained to discriminate differences in inbred mouse odor and then trained on pure odorants.

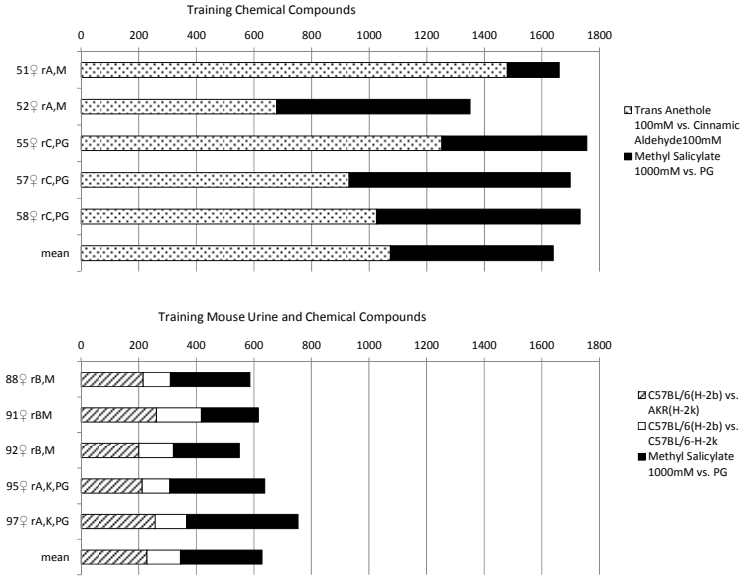


Fig 2. Training trials to criterion for two groups of mice trained to discriminate pure odorants in the Y-maze. One group (top) was trained directly on the pure odorants with no prior training on mouse odor. The second group (bottom) was first trained to discriminate mouse odor and then trained to discriminate pure odorants. Prior training on mouse odortypes facilitated learning to differentiate pure odors as shown directly by comparing trials to criterion for learning to discriminate methyl salicilate from PG (polyethelene glycol): black bars.

What we found was that training using mouse urine odor was accomplished more quickly than training on pure odorants (top vrs. bottom of Fig 2). This is consistent with the hypothesis mouse odors are easier to learn than non-mouse odors perhaps because the trained mice are more familiar with mouse odor and have had life-long experience with these odors. The results also suggest that training first on mouse odor facilitates later

learning of human odor differences as well as even differences among pure odorants. This is seen most clearly if we compare the black bars (trials to criterion on the task discriminating methyl salicilate from PG: mouse urine trained group faster than pure odor trained group, $p < 0.05$, Mann-Whitney U test). Perhaps mice are prepared to learn mouse odors. This "preparedness" may arise from the extensive prior experience mice have with mouse odor.

Several possible hypotheses can be suggested for these differences between greater ease of learning mouse odors compared with the learning human or pure odor differences. **First**, the age at which the animals were trained could account for the differences, at least as far as the comparison between learning mouse odor and learning human odor is concerned. As noted, the mice that were trained on human odor differences were older than the mice trained on mouse odor. There is evidence in mice and humans of diminished olfactory functioning with age and this explanation would be consistent with such an age-related change. **Second**, it may be that prior exposure to mouse odor during development underlies the rapid ability of the trained mice to discriminate mouse odors. **Third**, as noted above, the extreme difficulty in our first attempts to train mice to discriminate human urine samples could be due to the fact that the individuals randomly chosen as urine odor donors were of very similar HLA types. If this is the explanation it would be very strong and convincing evidence human MHC, like mouse MHC, is paramount for coding for odortype identity. These three explanations are not mutually exclusive. We next conducted studies to test these hypotheses.

Main studies

1. Age of trained mouse. We conducted training trials on mice of different ages to test the hypothesis that the trained mouse's age impacted its ability to learn in our Y-maze. For this experiment we trained 3 groups of mice which differed in age using only mouse urine odors. The results of these trials (Fig 3) indicated that older mice learned the task more slowly (Overall difference among the 3 groups, $p < 0.01$, Kruskal-Wallis test; 12 month old group slower than both the 5 week old group and the 6 month old group, $p < 0.01$, Mann-Whitney U test). Thus the difficulties we observed in training to discriminate between individual human urine odors could be due in part to a general difference based on the age of the trained mouse. However, this relatively modest age related difference in learning abilities cannot fully explain the difficulties in training mice to discriminate seemingly very different pure odorants as compared with training them to discriminate mouse urine odors. Thus age differences, while significant, do not seem to be the major explanation for the difficulties in training mice to discriminate between two humans as found in our pilot work.

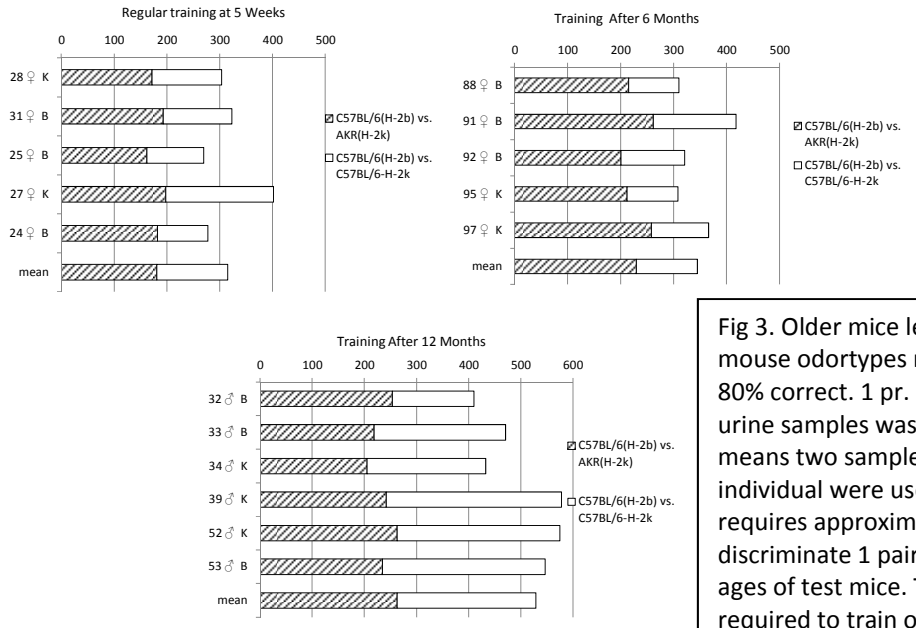


Fig 3. Older mice learn to discriminate mouse odortypes more slowly. Criterion = 80% correct. 1 pr. means a single pair of urine samples was used in training; 2 pr. means two samples from the same individual were used in training. Note that it requires approximately 200 trials tolearn to discriminate 1 pair of urine samples for all 3 ages of test mice. This is much less than required to train on individual human urine odor as shown in Fig 1.

2. HLA variation and prior experience with human odortypes. In the next series of studies, we focused on explanations 2 and 3 – whether more variation in the HLA types of the human individual pairs tested would facilitate learning and whether prior early experiences with components of human odortypes would predispose the trained mice to learn this complex task more quickly. In this study (Fig 4) we formed two panels of human donors such that each panel was identical at HLA A (either A3A3 [far left and far right for Fig 5] or A2A2 [center 4 humans in Fig 5]). Mice were first trained to discriminate between the urine odors of a pair chosen at random from the two panels (Fig 4 and Fig 5 top; HB41 vs. HB29). Following successful training the mice were then given generalization trials (see below for explanation of this procedure) with different pairings of individual urines from the two panels (connecting red and blue lines in Fig 5). The rapidity at which the trained mice discriminated between HB29 vs HB41 would test the hypothesis that major HLA differences are important for formation of human odortypes.

The other related issue tested was whether exposure to human HLA during development might also help mice learn to make human discriminations. During discussions with ARO visitors, we proposed to expose one group of mice to odors collected from human donors during early development of the mice that would subsequently be used in training and expose a second group to control exposures (presumable water although exactly what the control should be was difficult to determine; indeed several different kinds of controls would seem necessary). However, in follow-up discussions the suggestion was made that we could use transgenic mice that expressed a human HLA gene to vary exposure. The great advantage of this approach would be that the animals with the transgene would thereby be exposed to at least some aspect of human HLA-determined odor essentially from

conception and we would not need to decide how to accomplish the exposures; the mice would do this for us.

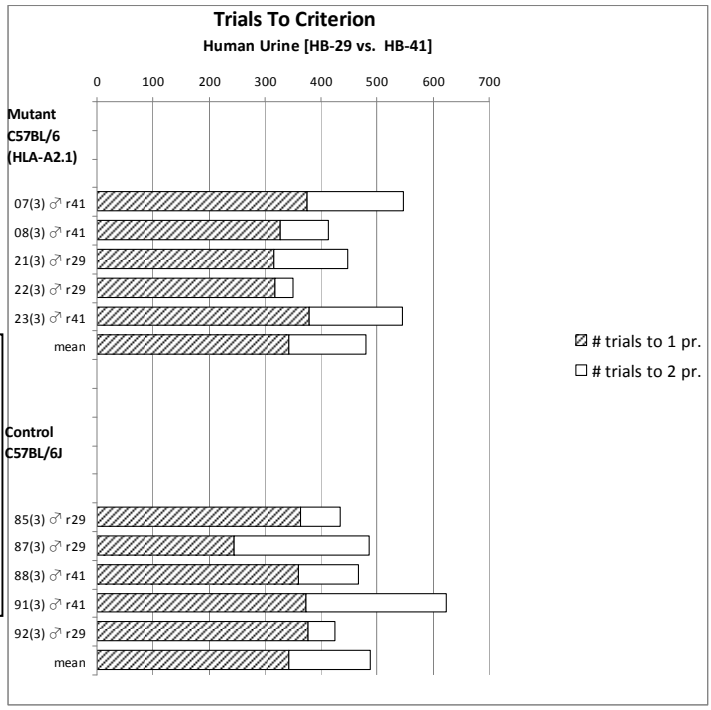


Fig 4. Study **2A**: Transgenic mutant mice and control mice learn to discriminate odors of 2 individual humans (HB29 vs HB 41) at the same rate. Criterion = 80% correct. 1 pr. means a single pair of urine samples was used in training; 2 pr. means two samples from the same individual were used in training.

We have thus conducted two related studies with HLA-A2 transgenic mice (on a C57BL6 [B6] background) and control B6 mice. First we trained 5 transgenic mice and in parallel 5 control B6 mice to discriminate between urine samples collected from two individual humans (Asians 23 and 24 years of age) that differed at HLA, one carrying an HLA-A2 specificity (HB29: HLA type A2A2 B27B62) and the other without this specificity (HB41: HLA type A3A3 B7B44). The two hypotheses tested (**Experiment 2A**) were (1) that these trained mice would learn to discriminate the individual urine samples much more quickly than the mice trained in pilot studies (Fig 1) because the two humans were much less similar at HLA and (2) that the transgenic mice, having had prior self-generated experience with HLA-A2, would learn the discrimination more quickly than the control mice.

The first hypothesis tested in experiment **2A** was strongly supported: The trained mice learned to discriminate between the two humans quickly compared with our pilot work (compare Fig 1 with Fig 4: more than 700 trials to criterion in the former and 300 – 400 trials in the latter). This result supports the hypothesis that greater HLA differences between individuals facilitates training. The second hypothesis of this study, that transgenic mice would learn more quickly than control mice because the former expressed human HLA A2 and thus were more familiar with components of human odor, was not supported. The transgenic and control mice learned to discriminate the two individuals at about the same rate (Fig 4 above; the means were almost identical).

Experiment 2B was a continuation of 2A in that the same two groups of trained mice were used. In this case they were tested with several different pairs of human urine donors in each case one of the pair carrying the HLA A2 allele and the other not carrying that allele.

The hypotheses tested were again two fold. Hypothesis (1) was that the trained mice would **generalize** (see just below for a description of this procedure and its interpretation) what was learned in experiment 2A with training on two individuals to other pairs of individuals with common differences. Hypothesis (2) was that the transgenic mice would do this more consistently that control mice.

Generalization procedure. We use generalization studies to test what the animal has learned during training. In generalization trials, mice are given a choice in the Y-maze between two samples that the trained mouse has never before encountered. In no case is the mouse rewarded for a response and in fact the operator of the maze is unaware of the nature of the two samples. The reasoning is that if under these circumstances the mouse chooses to go to the arm of the Y-maze that corresponds (as determined by the experimenters) to training, this proves that the mouse learned the class difference on which it was trained and not the specific individual. For example, mice are trained to discriminate male mice of different MHC types. Then in generalization trials they are given samples collected from females with the same MHC difference. If they successfully generalize in these unrewarded trials, we conclude that it was the MHC difference that the trained mouse learned to discriminate, not just something different about different male mice. In the current case, mice were trained as described above to discriminate between two individual male subjects that, among other things, differed in MHC type. In generalization trials the trained mice were offered choices between pairs of urine samples from other individuals that had the same MHC (HLA) difference. If they exhibited significant generalization we conclude that this demonstrates a role for HLA in human urine odors distinguishing different individuals.

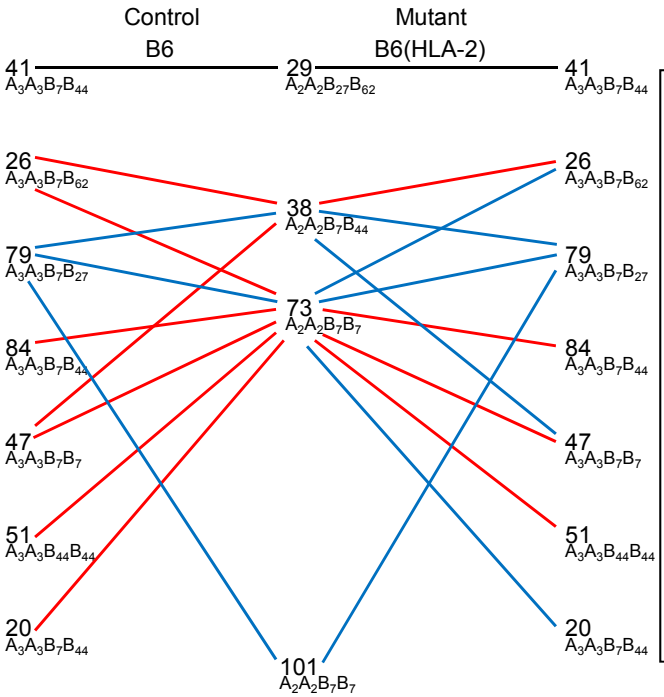


Fig 5. Study **2B**: Trained transgenic mice generalized more widely than control mice. Results of generalization tests of transgenic mutant and control mice with different pairs of individual human samples that differ in the presence or absence of an HLA-A2 specificity. For pairs of individuals connected by blue lines, generalizations were in the predicted direction and were statistically significant ($p < 0.05$; binomial test). For pairs connected by red lines, generalizations were not significant.

Testing the **first hypothesis in 2B** we found that the trained mice did generalize to some of the pairs although not all. This can be seen in Fig 5 where there were statistically significant

generalization scores in both sets of trained mice for several of the pairs (those connected with blue lines). This is quite remarkable since it is certain that these pairs of individuals differed greatly from the individuals who served as donors for the training samples. Thus in at least some cases the trained mice demonstrated that they had learned something about the HLA type during training. These data thus provide new and important data linking the prior mouse work to human odortypes and supporting the view that humans, like mice, can detect differences in HLA type based on differences in body odor. Testing the **second hypothesis in 2B** we found some evidence that transgenic mice did tend to generalize this learning to more pairs of other individuals (6 for transgenic [blue lines on right side], 3 for control [blue lines on left sides] out of a total tested of 10 pairs, one with an HLA-A2 specificity and one without this specificity; See Fig 5 above). However, more work is needed to confirm this conclusion with certainty.

Together, these results provide suggestive evidence that exposure to HLA-A2 parental, sibling and/or self odors influenced learning ability and they also imply that components of HLA-related human odors share similarities with odors controlled by these same genes when they are expressed in mice. Mouse odors and human odors under these conditions may have common components. To test this hypothesis we conducted the next experiment.

3. Mouse – human odortype similarities. Here we hypothesized that if HLA-A2 transgenic animals express odors in common with humans with HLA-A2 specificities, then mice trained to discriminate between two humans, one of whom carries HLA-A2 and the other that does not, should generalize this response to HLA-A2 transgenic mice verses control mice. Moreover, if experience with the HLA in mice is important, this should be seen particularly in trained transgenic mice. We tested this hypothesis with a new group of 5 young experimentally naive transgenic mice and 5 control B6 mice.

Table 1. Percentage concordance (no. of trials) of mice trained to discriminate two human urine samples (HB29 vs. HB41). Generalization trials in response to transgenic (HLA-A2) vs. control (B6) mouse urine odors.

Mouse number	Mouse urine generalization scores	
	first trials*	later trials
Control (C57BL/6)		
1	33 (15)	50 (10)
2	57 (14)	56 (9)
3	50 (8)	60 (15)
4	60 (10)	53 (15)
5	31 (13)	0 (4)
Total	**45 (60)**	**51 (53)**
Mutant (C57BL/6(HLA-A2)		
6	70 (10)	40 (10)
7	89 (9)	53 (15)
8	70 (10)	40 (10)
9	40 (10)	45 (11)
10	56 (9)	64 (14)
Total	**65 (48)****	**50 (60)**

* $p < 0.10$, Mann-Whitney U (transgenic vs control
** $p < 0.05$, binomial test (generalization of transgenic mice > 50%)

First, HLA transgenic mice and wild type control mice were trained to discriminate two individual humans. Here too, the training was relatively quick consistent with Experiment 2 results and contrary to the results of our pilot work. This provides further support for our belief that differences in the age at which mice are trained directly influences their ability to learn these complex differences between individuals.

Second, HLA-A2 transgenic mice trained to discriminate two humans differing in whether they carried an HLA-A2 specificity or not, significantly (65% correct; $p < 0.05$, binomial test) generalized this response during initial trials to the choice between urines collected from transgenic verses control mice. No such generalization was evident for trained control (B6) mice (45% correct). The difference between the behavior of the transgenic and control mice approached significance ($p < 0.10$, Mann-Whitney U test). It should be noted that in later trials this significant generalization by the transgenic mice was no longer evident (50% for transgenic mice, 51% for control mice). We believe that this is because during these generalization trials the mice began to learn that they would not get rewarded when mouse urine was tested (mouse and human urine odors are very easy to discriminate even by the human investigators). Due to an inability to obtain more HLA-A2 transgenic mice within the confines of the grant period we were not able to follow up on this suggestive result by replicating and extending it. Our intent is to try to pursue this with other sources of support.

Related findings not in the originally proposed work.

After we began the work described above, we conducted pilot studies that suggested that mice that had had been immunized developed a novel odor that could be detected by other mice. Our original idea was that this could provide another tie between immune function and body odor and enhance our understanding of differences between human odors (where immune function status surely differs between different individuals) and mouse odors where this variable is kept constant. Thus we proposed to the program officials in early correspondence and meetings that we would like to follow up on this work with ARO support. However, the program officials for this grant thought that this was moving too far from the original proposal and thus indicated that we should not pursue this work with support from these ARO funds. We complied with this decision but since the initial work, conducted prior to discussions with the program officers, was done with this support we think it valuable to provide a very brief follow-up on these investigations. They were supported by internal Monell funding first and subsequently we received an ARO grant to pursue the work which is ongoing.

Briefly, in a series of bioassay sessions using a Y-maze apparatus, we trained mice to distinguish between urine odors of rabies-vaccinated (RV) mice compared with mice given injections of vehicle only (control). RV-trained mice generalized this training to the choice between mice immunized with the equine West Nile virus (WNV) vaccine compared with urine of corresponding controls. These results suggest that there are similarities between body odors of mice immunized with these two vaccines. To further investigate specificity of odors induced by immune activation, a second RV-trained biosensor panel was was tested in generalization trials only with a choice between urine from donors treated with lipopolysaccharide (LPS; a general elicitor of innate immunological responses) compared to vehicle controls. RV-trained biosensors did not distinguish between LPS-induced compared with control odors. Finally, we directly trained mice to discriminate between LPS and control urine odors in a third biosensor panel. In generalization tests, these trained mice

distinguished between urine samples from LPS-treated compared with RV-treated mice for samples collected less than 12 days post-treatment. We conclude that immunization alters urine odors and that these alterations persist for many days following their induction. Furthermore, odor changes resulting from LPS (an innate inflammatory agent) differ from odor changes induced by vaccinations which elicit adaptive immune responses. We are following up on this work now with ARO support (W81XWH-12-2-0081, EDMS 5584; G. Beauchamp, PI) using both behavioral and chemometric approaches.

Project 4: Plasticity in olfactory bulb neural networks

Graeme Lowe

Executive summary

Synaptic plasticity in olfactory bulb circuits is a potential substrate for olfactory memory. NO is an intrinsic neuromodulator in the bulb and it has been hypothesized to function in olfactory memory. In this project, we investigated cellular mechanisms of NO function by electrophysiological recording of NO modulation of neuronal and synaptic activity in the bulb. We found that NO exerted measurable effects at every stage of bulb signal processing. It facilitated synaptic transmission at olfactory nerve terminals, accelerated the activity of glomerular pacemaker (ET) cells, and increased excitatory synaptic drive and spike firing in mitral cells. Conversely, NO could also engage inhibitory synaptic pathways which control timing and synchronization of excited mitral cells. We hypothesize that well coordinated amplification of activity and synaptic transmission in mitral cells could promote sparsening and synchronization of odor representations encoded as mitral cell spike trains. This could be the means through which NO can facilitate synaptic plasticity (LTP/ LTD) in bulb circuits or downstream targets in piriform cortex. A parallel effort in this project was the technical development of a new method of light sheet confocal imaging (OCPI) for application in brain slices. We completed the engineering and initial testing of this apparatus and developed protocols to load calcium indicators into neurons in olfactory bulb slices. We have set the foundation and acquired the preliminary data to pursue future more detailed studies of NO and odor memory. We plan to systematically map connectivity in bulb slices stimulated by NO and trace cellular pathways of synaptic modulation that we have recorded mitral cells. This work will increase our knowledge about cellular mechanisms underlying odor memory.

Background and Objectives

A fundamental problem in olfaction is to identify cellular mechanisms of odor learning and memory. Memories of odor experiences may be stored as durable changes in the physiology or architecture of central neural networks. Synaptic connections in networks may be altered by long term potentiation (LTP) or depression (LTD), and neuronal populations may increase or decrease with neurogenesis or cell death. Evidence for neural plasticity has been reported in different stages of the olfactory pathway, from peripheral olfactory receptors to the olfactory bulb and piriform cortex. The olfactory bulb contains a diversity of neurons and abundant synapses that could undergo changes during odor learning. Odor-encoding activity patterns received by olfactory bulb glomeruli are relayed to bulb output neurons (mitral/ tufted cells). Inhibitory dendrodendritic synapses from local interneurons (PG/ granule cells) filter and reshape activity patterns of mitral/ tufted cells. During odor learning, long term changes in strength and connectivity of these synapses may alter odor-specific patterns of mitral/tufted activity, leading to long term changes in odor perception. Alternatively, odor training may induce short term changes in bulb activity patterns, increasing sparseness or synchronization in firing patterns of bulb output neurons, which can lay down or reinforce odor memories in downstream networks of pyramidal neurons in olfactory cortex. Changes in olfactory bulb neural circuits that underlie odor learning have not been well delineated. Studies have found olfactory learning is associated with actions of certain neuromodulators – e.g. acetylcholine, noradrenaline, nitric oxide (NO) – on olfactory bulb circuits during odor conditioning.

Our specific aims have been refined as follows: *Aim 1*: To develop and apply new calcium imaging methods to study plasticity in mouse olfactory bulb circuits at the level of functional neural networks. Electrophysiology has been used to characterize synaptic plasticity (e.g. LTP) in one or a few cells at a time, or as average changes over cell populations. Calcium imaging can assay activity patterns across many cells, and potentially map large scale plasticity and rewiring of connections in olfactory bulb networks. *Aim 2*: To determine how activity patterns of bulb neurons are altered by the gaseous neuromodulator, NO, which is strongly expressed in the olfactory bulb and has long been implicated in odor learning.

Approaches

We use in vitro olfactory bulb slices, which allow high resolution live cell imaging and well controlled pharmacological manipulations. *Aim 1*: In calcium imaging experiments, cells in slices are bulk-loaded with fluorescent indicator dye, Calcium Green. Fluorescence signals report changes in intracellular calcium indicating firing of action potentials. To search for changes in bulb circuits, we can test connectivity either: (i) by mapping PG/ granule cells that respond when a selected mitral cell or glomerulus is electrically stimulated (Trigger-Follower), or (ii) by correlating PG/ granule cell calcium signals with mitral cell inhibitory synaptic potentials (IPSCs) (Reverse Optical Trawling). As connections may be sparse, we seek to increase the number of sampled cells by sweeping the plane of focus through multiple layers of cells. This will require adapting the new technique of Objective Coupled Planar Illumination (OCPI) to the olfactory bulb slice. *Aim 2*: Examine modulatory effects of NO on the activity of various olfactory bulb neurons by targeted electrophysiological recording. Effects of NO on overall activity patterns are assayed by calcium imaging. Our long term goal will be to combine this with connectivity assays (Aim 1) to test if NO causes transient or long term changes in stimulus-evoked patterns of PG/ granule cell response.

Progress

Aim 1: Calcium imaging method: (1) Engineering new technology: we have completed development and construction of a functioning rig for high speed calcium imaging in brain slices by OCPI method; (2) Developing calcium imaging in olfactory bulb slices: a working protocol was developed for Calcium Green-1 indicator loading into cells in the olfactory bulb slice preparation by ester cell permeation of AM ester dyes. PG and granule cells were loaded in slices, and tests we done to show the cells remained physiologically viable. We also started to develop image data analysis routines to track calcium signals in cell populations. *Aim 2*: We recorded changes in neural activity in olfactory bulb neurons induced by NO: (1) increased firing of neurons in the glomerular layer (PG or tufted cells) and mitral cells; (2) changes in synaptic activity in mitral cells.

Results

Aim 1: (1) OCPI system engineering: we report the completion of assembly of all key components of the OCPI calcium imaging apparatus, and system testing:

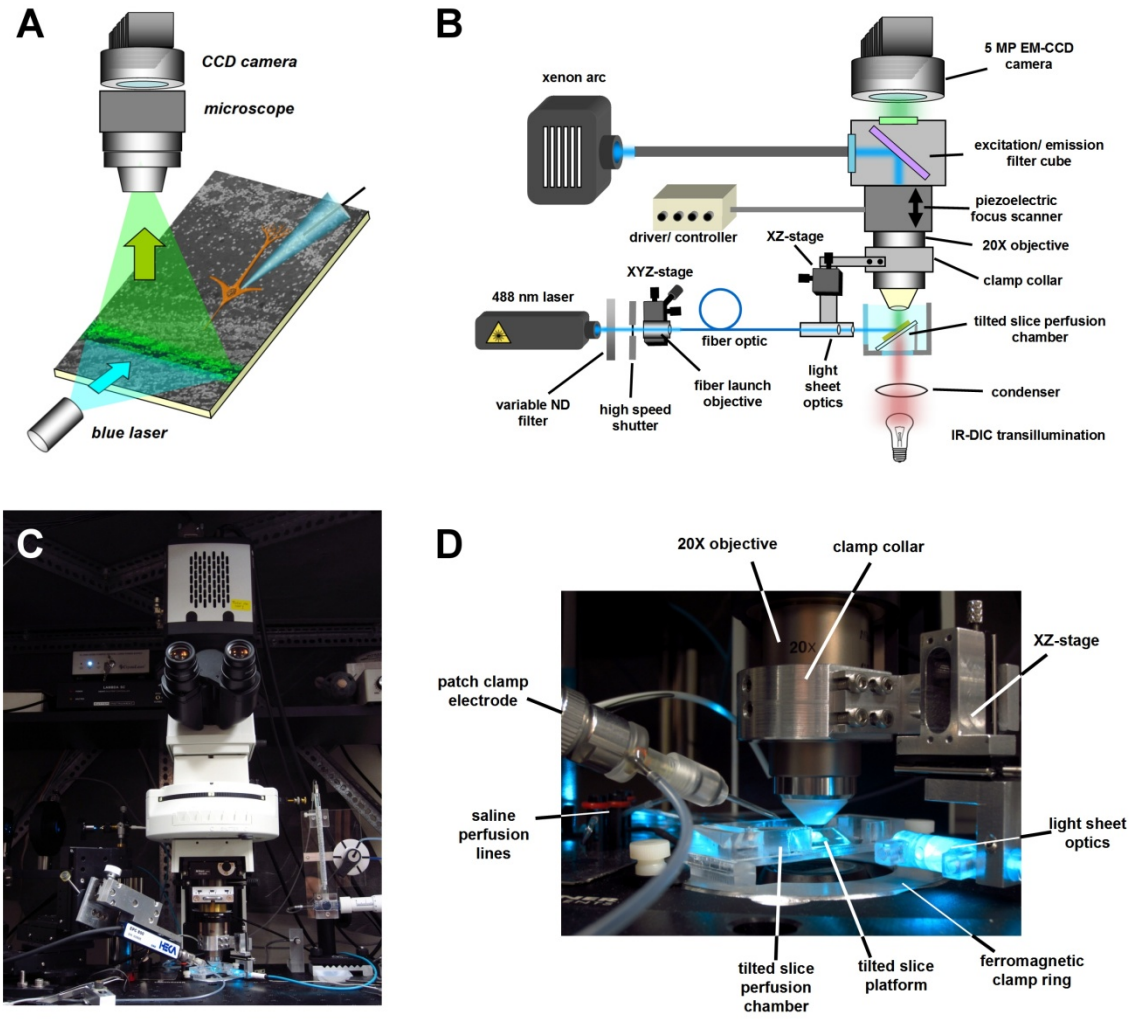


Figure 1. A. Schematic of OCPI concept for vertical-scanning confocal laser fluorescence microscopy in olfactory bulb slices (light sheet excitation). **B.** Schematic of design implementing OCPI in slice preparations. Illumination, scanning, image acquisition and electrophysiology are coordinated by two computers. **C.** Complete calcium imaging rig with OCPI. **D.** Close-up of slice chamber, with blue laser light-sheet illumination of tilted slice platform through lateral window of perfusion chamber. In OCPI, light sheet optics and objective are coupled, moving in tandem during focus scanning by a piezoelectric transducer.

Aim 1: (2) Calcium imaging: A dye stock solution was made (10 mM Calcium Green-1 AM ester in DMSO + 20 % pluronic F-127), diluted to 1 mM in saline, and 40 µl applied to slices in an oxygenated interface chamber for 50 min. Glomerular layer neurons internalized and trapped the dye and responded to depolarization by fluorescence increases (Fig. 2). This demonstrated a viable preparation for stimulation and imaging studies.

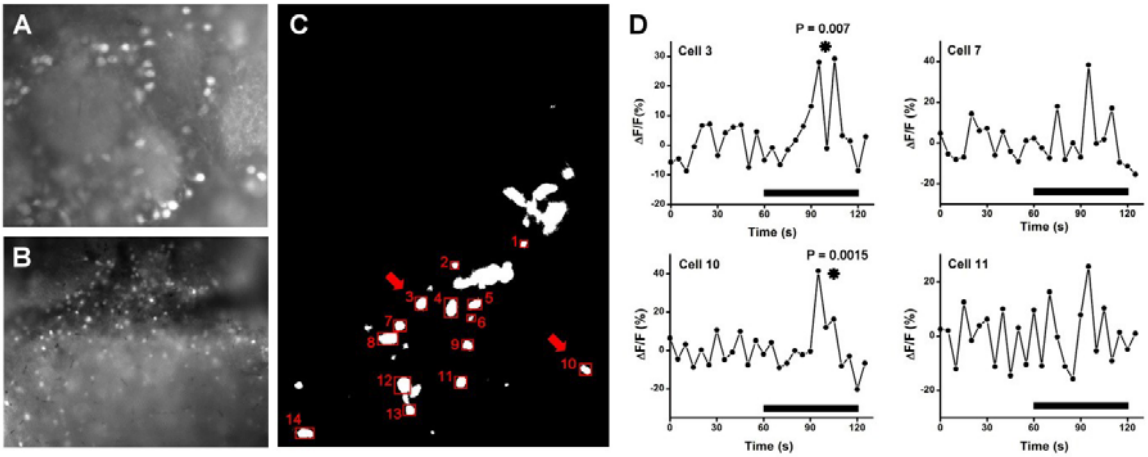


Figure 2. A-B. Calcium Green-1 loaded cells in glomerular and sub-glomerular layers of mouse olfactory bulb slices. **C.** Fluorescent cell bodies analyzed Regions of Interest (ROI 1–14) defined by intensity threshold. **D.** Tracking of ROI mean fluorescence over time for 4 cells in C. Black bar: time of depolarization by 100 mM K^+. Asterisk: peak response significantly above baseline fluorescence.

Aim 2: Actions of nitric oxide (NO) on olfactory bulb neurons: (1) *NO excites external tufted (ET) cells*: Recordings of spike activity in neurons of the glomerular layer revealed an excitation by the NO donor NOC7 in 4/9 tested cells. The stereotypic bursting patterns of activity indicated that these cells were in fact ET cells. The ET cell bursting was accelerated and burst duration was shortened, demonstrating a clear modulatory action of NO on glomerular activity (Fig. 3).

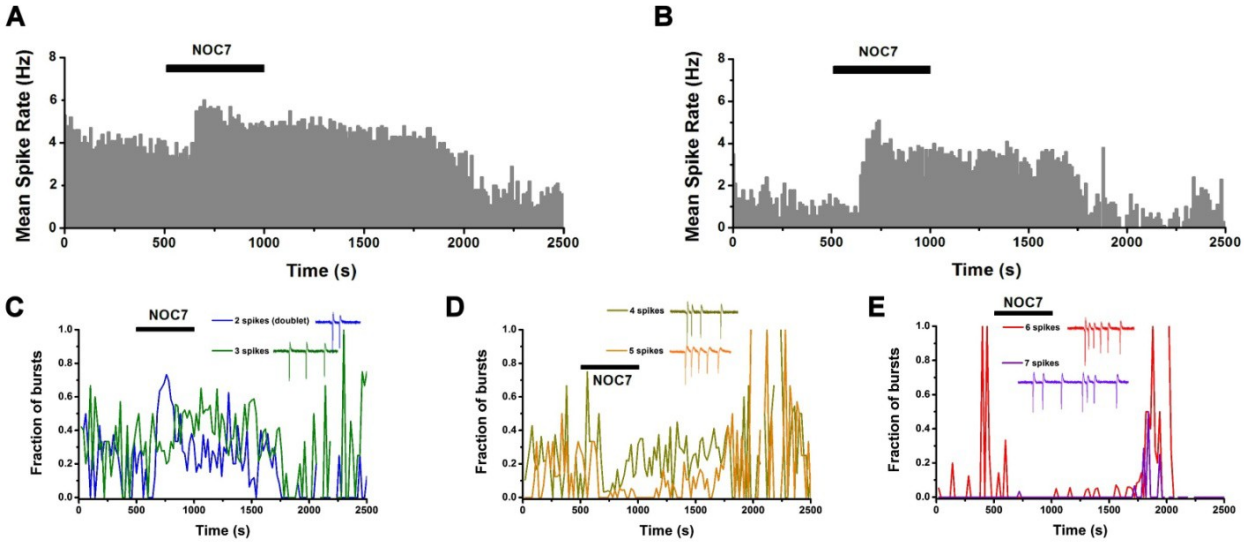


Figure 3. Physiological effects on ET cells of applying NO with 100 µM NOC7 (NO donor). A-B. NO increased the mean spike rate in ET cells. **C-E.** NO increased the fraction of short duration bursts (N = 2 spikes; doublets) and decreased the fraction of long duration bursts (N > 3 spikes).

(2) *NO exerts both excitatory and inhibitory effects on mitral cells* (Fig. 4): recordings from mitral cells revealed both slow inward and outward currents, and increases in both excitatory and inhibitory synaptic inputs caused by the NO donor NOC7 (data from 2 cells). Modulation of opposing synaptic activities could occur together in the same cell (c.f. Fig. 4D).

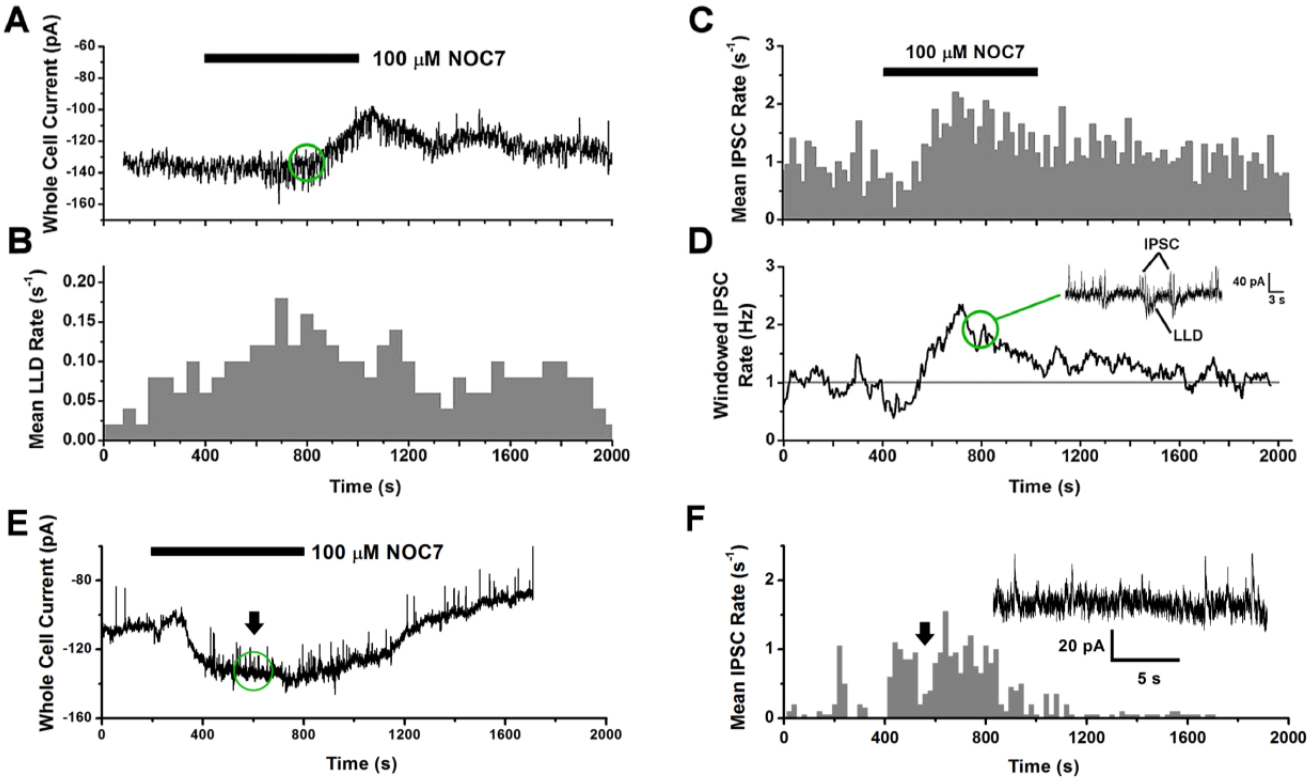


Figure 4. Physiological effects on mitral cells of applying NO with 100 µM NOC7 (NO donor).A. NO induced a slow outward (hyperpolarizing) current in a mitral cell. **B.** NO increased the mean rate of slow excitatory postsynaptic currents (EPSCs) known as long lasting depolarizations (LLDs). **C.** NO increased the mean rate of fast inhibitory postsynaptic currents (IPSCs). **D**. IPSC rate plot from C. Green circle/ inset: expanded trace showing IPSCs were correlated with LLD-type EPSCs. **E.** NO induced a slow inward (depolarizing) current in a mitral cell. **F.** NO increased the mean rate of IPSCs in the mitral cell shown in E. Inset: expanded trace showing IPSC events (at time indicated by arrows/ green circle in E & F).

(2) *Tonic modulation of neuronal activity and synaptic signaling by NO in olfactory bulb neurons* (Fig. 5): We previously reported evidence for basal endogeneous production of NO in the olfactory bulb that may regulate neuronal activity (Lowe et. al. 2008). To further

investigate the roles of tonic NO, we recorded the effects of blocking NO tonic signaling on ET/ PG and mitral cells. We found that the NO scavenger cPTIO could: (i) both increase and decrease the spike activity of mitral cells; and (ii) decrease the strength of synaptic input received by ET/ PG cells from olfactory nerve inputs. This indicated that even the intrinsic activity generated in bulb circuits was subject to widespread and diverse regulation by NO signals.

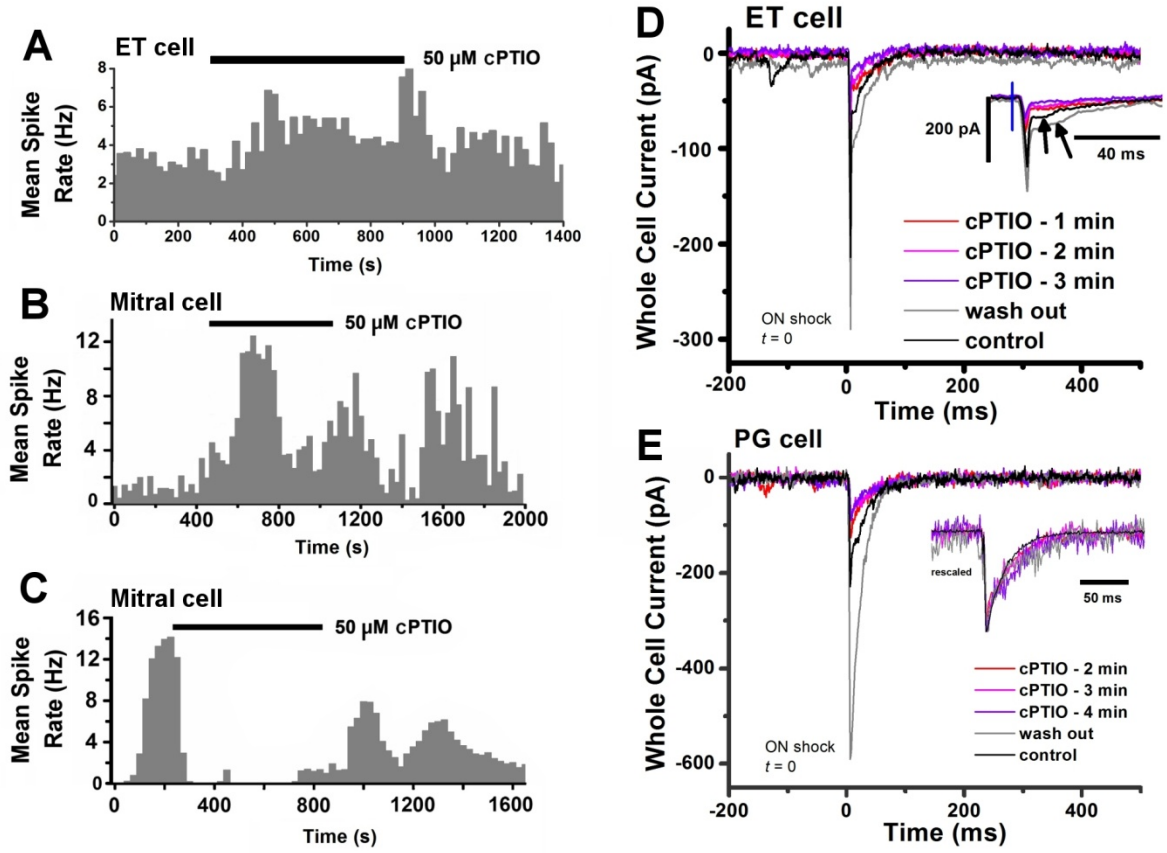


Figure 5. Effects of blocking NO signaling with 50 µM cPTIO (NO scavenger). A. An ET cell was excited and increased its spiking frequency. **B.** A mitral cell also showed excitation, by increasing its spike rate **C.** However, spiking in another mitral cell was decreased by NO block. **D.** The EPSC evoked by shocking sensory input to the bulb (olfactory nerve terminals) was reduced by NO block. **E.** Similar phenomenon in a PG cell. Invariance of time course of reduced EPSCs in both cell types indicates NO up-regulates transmitter release (glutamate) from a common input, i.e. the sensory nerve terminals.

Significance:

Synaptic plasticity in olfactory bulb circuits is a potential substrate for olfactory memory. NO has been hypothesized to function in olfactory memory. Our study revealed widespread effects of NO on neuronal activity and synaptic transmission at every stage of olfactory bulb processing. At the sensory input stage, NO apparently facilitates glutamatergic transmission at olfactory nerve terminals. At the early glomerular stage, NO accelerates ET cell burst firing and shortens bursts, promoting collective excitation of glomerular networks

of principal cells. This is reflected in an elevated rate of LLD-type EPSC activity in mitral cells. Observation of a slow inward current also suggested a direct depolarizing action on some mitral cells. On the other hand, we also observed enhance of parallel inhibitory synaptic pathways by NO in some mitral cells. This may reflect the complex, negative-feedback architecture of bulb circuits. Since NO is synthesized and released within the bulb, it is an intrinsic modulator that may operate in conjunction with extrinsic conditional modulators to trigger long term changes for odor learning and memory. Intensification of activity and synaptic transmission in glomeruli and mitral cells is expected to promote sparsening and synchronization of spike activity in mitral cell odor representations. From our data, we hypothesize that NO facilitates synaptic plasticity in the olfactory bulb, or downstream piriform cortex where odor information encoded by different glomeruli is integrated. Strong NO-modulatory effects we recorded on EPSCs/ IPSCs in mitral cells should be detectable by applying the OCPI calcium imaging system we constructed to more systematically map synaptic connectivity in bulb slices stimulated by NO.

Project 5: Olfactory cues for stress reduction in a military population

Pamela Dalton and Johan Lundström

Executive Summary

We proposed to conduct two studies to better understand how social odors (body odors from self, sibling or stranger) could modulate stress as measured by self-report and autonomic arousal indices (heart rate, skin conductance, stress hormones). We also sought to confirm that body odors from strangers could activate brain structures involved in fear and arousal (amygdala) and that this response would be enhanced among individuals with high levels of social anxiety. Results from the first study confirmed that the body odor of a sibling promoted faster recovery from stress than did the non-social odor (gardenia) or the body odor of a stranger. In fact, exposure to the body odor of a stranger prevented recovery from stress among most individuals. Results from the second study show that exposure to the body odor of a stranger activates the limbic system, specifically the amygdala and that this effect is enhanced among individuals exhibiting high levels of social anxiety. The findings may point the way toward non-pharmacologic therapies aimed at reducing stress, promoting vigilance and treating social phobias.

Background and Objectives

There is a current need in the military for more non-pharmacological approaches to the treatment of stress following deployment. Olfaction plays a unique role in how we gather and process information of our surroundings and circumstances and may offer therapeutic options for treating stress in both field and garrison settings. It is acknowledged that olfactory cues have the potential to precipitate memories with strong emotional components[26]. In prior work, we demonstrated that exposure to a novel odor while undergoing a laboratory stressor caused individuals to re-experience stress (increased heart rate & self-reported stress) when re-exposed to that odor three days later[27]. Interestingly, pre-exposure to the novel odor in a non-stressful context prevented the stress-odor association from forming.

Among individuals with PTSD, olfactory cues can prompt re-experiencing of emotional trauma[28-30]. One class of olfactory stimuli which has shown promise in eliciting robust effects on mood and emotion is social odors. Animal studies have demonstrated clear evidence of hard-wired fear responses to predator odors[31] in the absence of prior experience. Similarly, Lundström et al.[32] recently demonstrated that smelling a stranger's body odor activated cerebral regions similar to those found to be active when viewing perceptually masked fearful faces[33]. Despite a low level of conscious recognition of the body odor's source, a marked response in the amygdala of all participating subjects was noted.

Study 1 Goal: We conducted a study to determine whether social odors (body odors from a close relative) reduce the stress and anxiety elicited by an experimental stress task better than the body odor of a stranger or a non-social odor purported to be 'relaxing'.

We first collected body odor from two siblings to be used as the 'stimuli' in the subsequent experiments. Axillary odors were collected from T-shirts worn overnight by the participants, brought into Monell and frozen. During the experiment, each member of the sibling pair was tested individually.

Progress to Date

We have completed testing for the behavioral study with 24 participants tested in the Sibling Odor group (17 Females), 24 participants tested in the Stranger Odor Group (11 Females), and 22 participants tested in the Non-Social Odor Group (14 Females).

In this first study, stress was induced by using the Trier Social Stress Test (TSST) and measured by ratings of stress and anxiety, stress hormone levels (salivary cortisol and alpha amylase) and psychophysiological recordings (heart rate and skin conductance). After the stress- induction, participants were exposed to the underarm odor of a sibling (Sibling Odor Group), the underarm odor of a stranger (Stranger Odor Group), or the fragrance of a non-social odor [Gardenia] (Non-Social Odor Group).

We hypothesized that stress reduction is enhanced for the group exposed to their siblings' body odor when compared to the stress reduction in (1) the group receiving the stranger odor, (2) the group receiving non-social odor and (3) a control group receiving no odor (data already collected).

Results

Psychophysiological Recordings

Heart Rate:

All groups showed a significant increase in Heart Rate (HR) during the TSST, and a significant decrease in heart rate by the end of the recovery period. However, only the heart rate for the participants in the Sibling Odor decreased significantly below baseline, suggesting a benefit on stress reduction that was greater in this group than the other groups. (See Fig 1).

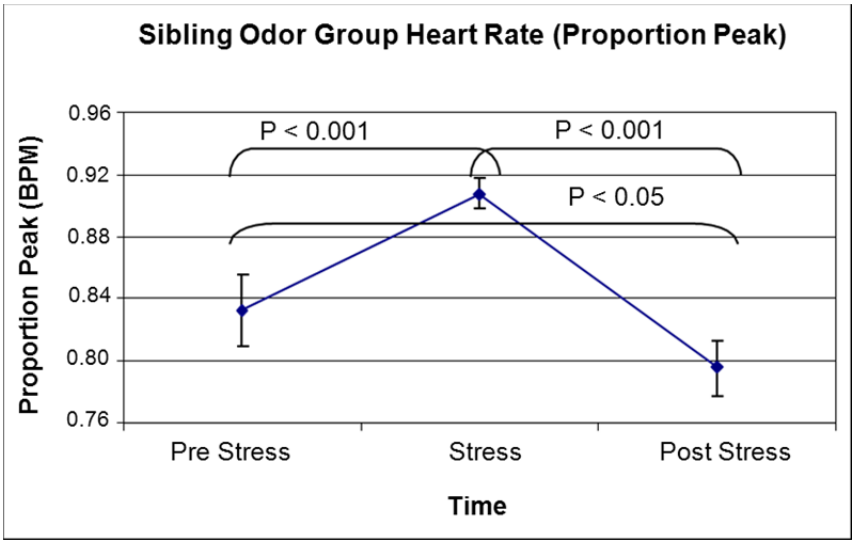


Fig. 1. Sibling Odor Group Heart Rate

Skin Conductance:

Skin conductance, a measure of autonomic arousal, increased significantly during the TSST for all groups, affirming the success of the TSST manipulation. However, only the Stranger Odor Group did not decrease significantly during recovery. In contrast, both the Sibling Odor Group and the NonSocial Odor Group decreased significantly from their highest peak response (See Figs 2 A, B, C).

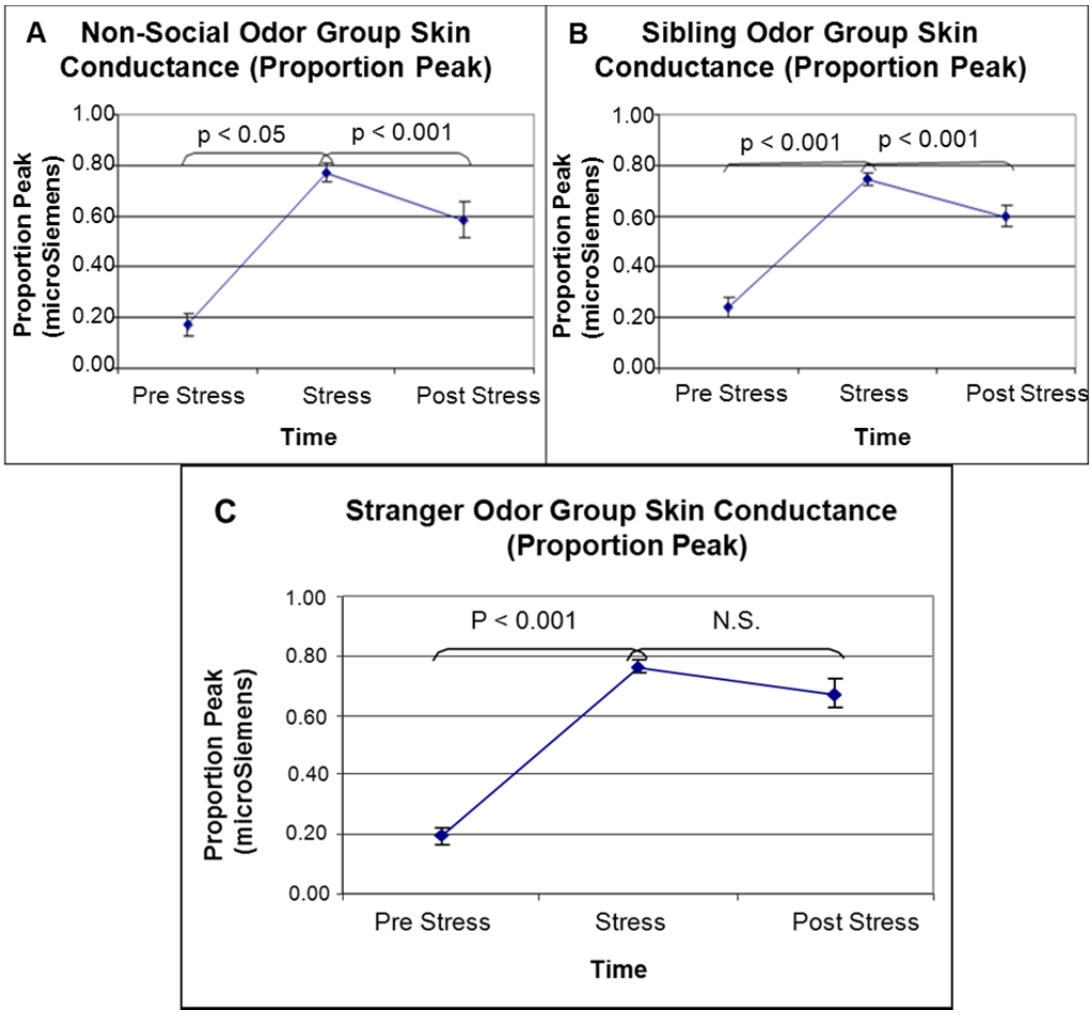


Fig. 2. Skin Conductance for A) Non-Social Odor Group B) Sibling Odor Group, and C) Stranger Odor Group

Self-Report

The Self Report measures we collected show, in general, no pattern and at times contradict each other and the Objective endpoints we collected, thereby highlighting the frequent finding that self-report is dissociated from other measures of physiology and functioning.

Visual Analog Scales (VAS):

While the psychophysiological endpoints demonstrated a clear stress response and recovery, when making VAS ratings about Anxiety and Stress, participants' subjective ratings seem to contradict their objective endpoints.

Anxiety VAS ratings, for example, showed a significant increase for the Sibling Odor and Non-Social Odor Groups, but not for the Stranger Odor Group. Significant decrease from stress was illustrated in the Non-Social Odor Group but not the other groups.

Stress VAS ratings, on the other hand, showed a significant increase for the Stranger Odor and Non-Social Odor Groups, but not the Sibling Odor Group. Significant decreases from stress were observed in the Stranger Odor Group but not the other groups.

Profile of Mood States (POMS):

Anxiety Scores for the POMS showed a significant increase for all groups during the TSST, but the scores only decreased significantly for the Non-Social Odor Group.

Diagnostic Adaptive Behavior Scale (DABS):

DABS scores for Anxiety showed a significant increase for the Non-Social Odor and Sibling Odor Groups during the TSST, and decreased significantly for the Non-Social Odor and Stranger Odor Groups. Conclusions

As hypothesized, we found that the subjects experiencing the 'sibling' odor post-TSST exhibited greater stress reduction, as measured by heart rate, than did the subjects experiencing the 'stranger' odor or the non-social odor. Although none of the groups returned to their pre-stress baseline on the skin conductance measure, the 'sibling' odor group and the non-social odor group did show significant reduction on this measure, whereas those experiencing the 'stranger' odor did not. It would appear from these data that the body odor of a 'stranger' serves as an arousal cue to heighten vigilance, and when experienced in an already stressed state, may serve to maintain or even amplify the stress level. Whether this arousal cue has utility for maintaining vigilance or attention under various circumstances remains to be investigated.

Study 2 Cerebral response to body odors from self vs. stranger

Goal: Because we observed heightened activation on several physiological endpoints when individuals were presented with the body odor of a stranger, compared to the body odor of a sibling, we wondered whether this effect would be enhanced among individuals who were high in social anxiety and whether this effect would be evident in brain structures involved in anxiety or fear response (i.e. amygdala). Social anxiety, also called social phobia, is an anxiety disorder in which a person has an excessive and unreasonable fear of social situations, which we hypothesized could be triggered by exposure to a stranger's body odor.

Subjects: We recruited 30 participants, 15 high in social anxiety and 15 low. Other inclusion criteria were non-smoking and right-handed.

Study design: We used a 2 (Group; hi vs low social anxiety) X 3 (Odor: self, stranger, clean air) X 3 (stimulus: angry, neutral or scrambled face) study design.

Procedure: All subjects were given clean t-shirts to wear for 2 nights in order to collect their body odor. One t-shirt odor would be used in the 'self' odor condition, the other t-shirt odor would be used for another subject in the 'stranger' condition. Rapid echo-planar imaging using a mixed event-block design was conducted on participants while they were being exposed to all three odors in a randomized order, using a Philips Intera Achieva 3.0 Tesla scanner with a 32-channel head coil. While being presented with the odor, they were also asked to rate the emotional quality of the three types of faces, as previous studies have shown that individuals exposed to body odors obtained from stressed individuals rate neutral faces as more negative or angry.

Results: All data were analyzed using a multivariate flexible-design statistical model within the SPM software package. Final results are still being analyzed, but the primary finding is that individuals who are high in 'social anxiety' exhibit increased activation in the amygdala region when smelling the 'stranger' odor, when compared to individuals who are low in 'social anxiety' ($p<0.01$, Fig 3). This group effect suggests that social odors of a stranger elicit anxiety and fearfulness among individuals with high social anxiety, consistent with our hypothesis.

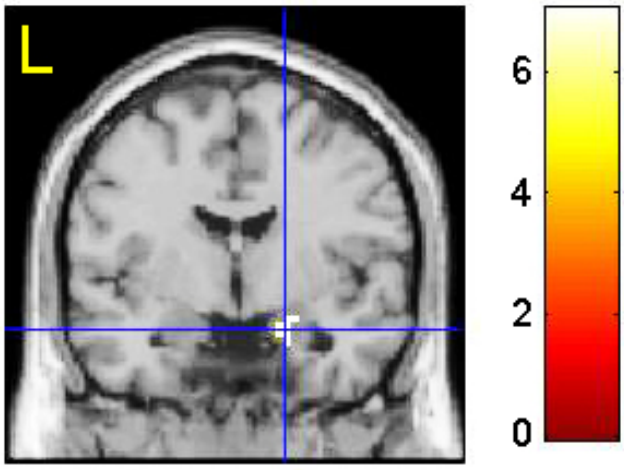


Figure 3. Group effect of (n=12) response during 'stranger' odor showing contrast activation of amygdala region in subjects with high social anxiety.

Project 6: Experience-dependent modulation of human olfactory function

Beverly Cowart, Johan Lundström, Marcia Pelchat and Kathrin Ohla

Executive Summary

We proposed a study to examine the effects of intermittent exposure to complex odors on olfactory sensitivity/processing in young adults. Specifically, we sought to assess the effects of active cognitive engagement (CE) during exposure *versus* mere exposure (ME) via both electrophysiological, olfactory event-related potential recordings (OERP), and behavioral measures. Our electrophysiological results show learning dependent amplitude changes at two levels with intermittent odor exposure: the early negative (N1) and late positive components (LPC) of the OERP, with effects being most pronounced following CE exposures. ME yields similar, though somewhat weaker, effects at N1, but none at the higher perceptual/cognitive level of LPC. Behavioral (threshold) measures are generally consistent with the OERP data in showing improved sensitivity. However, these sensory measures did not significantly differentiate between CE and ME groups, and may largely reflect the N1 response. These findings suggest that the distal perception of environmental dangers may be enhanced in Army personnel through repeated exposure, and point to active cognitive engagement during exposure as a means of strengthening that enhancement.

Background and Objectives

Olfaction is a distal sense that, although under-appreciated, provides critical information about surrounding dangers as well as about possibly useful/pleasurable elements in the environment. There is considerable evidence that olfaction is, in many ways, a “learned” sense, evidencing experienced-induced plasticity in both central circuits and peripheral receptors even in adulthood. A number of studies on adults have shown that periodic exposure to an odorant under a variety of conditions can lead to improvements in olfaction[34-41]The types of subject differed across studies: some were human, some animals; some had specific or general olfactory deficits and others did not. There were also differences across studies in depth of processing (i.e., simple sniffing versus a discrimination task). Yet, they all produced improvements in olfactory sensitivity. In short, considerable evidence indicates that olfactory abilities can be enhanced by systematic exposure to odors. This is of interest to the Army as a way of enhancing distal perception of dangers for its personnel and, perhaps, of counteracting declines in olfactory sensitivity associate with aging[42, 43] or head trauma[44, 45].

The aims of this study were to (1) determine, via both electrophysiological and behavioral measures, if repeated exposure leads to enhanced responsiveness to complex odors occurs in young adults with normal olfactory function; (2) determine which of two approaches to olfactory exposure produces more change in olfactory function/processing: mere exposure to olfactory stimuli (ME) or exposure with active efforts to identify/discriminate the stimuli (cognitive engagement: CE); and (3) determine, via electrophysiological measures, the extent to which peripheral (sensory) and/or central (perceptual/cognitive) mechanisms underlie changes.

Approach

The odorants used were complex mixtures that mimic or are derived from natural odor sources (wintergreen, banana, anise, bubblegum, orange and peanut butter). Subjects received two jars of each of the first four odorants to be sniffed twice daily throughout the study period; half of the subjects were instructed to simply sniff the odors (mere exposure: ME), and half practiced identifying the odors (sorting the 8 jars by odorant name) each time they smelled them (cognitive engagement task). The remaining two odorants served as controls, to assess the specificity of changes in olfactory sensitivity with exposure. Measures of sensitivity obtained for all six odorants included olfactory detection thresholds and, for two of the exposed odorants (banana and anise) and for both unexposed odorants, olfactory event-related potential recordings (OERP) and recordings from external electrodes placed along the nasal midline. Wang et al.[46] suggested that the latter reflect olfactory receptor responses from the epithelium (the so-called electro-olfactogram: EOG). Although our data indicate these electrodes do reflect a distinct and possibly more peripheral response than the ones reflected in OERP, we believe the signal source likely originates from the olfactory bulb, and thus refer to it as an electro-bulbogram (EBG); we are currently conducting independent studies in an attempt to confirm the source of this signal. Table 1 provides an outline of the study design.

	Mere exposure (ME)		**Cognitive engagement (CE)**	
Session 1	Thresholds, OERP & EBG at Monell Begin sniffing odorants 2x/day at home		Thresholds, OERP & EBG at Monell Begin sniffing& identifying odorants 2x/day at home	
Weeks 1-6	Sniffing odorants 2x/day at home		Sniffing & identifying odorants 2x/day at home	
Session 2	Thresholds at Monell		Thresholds at Monell	
Weeks 7-12	Sniffing odorants 2x/day at home		Sniffing & identifying odorants 2x/day at home	
Session 3	Thresholds, OERP & EBG at Monell		Thresholds, OERP & EBG at Monell	
Weeks 13-18	Stop sniffing	Continue Sniffing	Stop sniffing	Continue sniffing
Session 4	Thresholds, OERP & EBG at Monell		Thresholds, OERP & EBG at Monell	

Table1. Study design

Results

Subjects. A total of 64 subjects were enrolled in the study. Forty subjects completed the major portion of the training/exposure trial (sessions 1-3—baseline to 12 weeks); 38 of those were also able to complete the final 6-week follow-up Seven subjects were dropped from the study due to the absence of a clear OERP response (although all could detect the

odorants; see comment below). An additional 2 were initially so sensitive to multiple odorants that shifts toward greater sensitivity would not be detectible with our threshold series, and 1 subject's hairstyle prevented OERP recording. Finally, 6 were lost to follow-up, 5 withdrew voluntarily for personal reasons, and 3 were withdrawn because of failure to maintain the study protocol. Subjects who completed at least the first 3 sessions included 21 males and 19 females, and ranged in age from 19-50 years (median = 25 years). The representation of racial/ethnic groups is: non-Hispanic Caucasians= 28, Hispanic Caucasians=2, non-Hispanic Blacks=4, Asians=5 and Other=1.

OERP and EBG recordings. As noted, several subjects failed to yield clear OERPs in response to odorant stimulation. This is not surprising given that OERPs are relatively weak and noisy compared to visual and auditory ERPs , where tighter control of stimulus onset and offset is possible. No consistent basis for these failures is obvious; however, minor variations in head shape, hair quality and/or fitting of the electrodes likely contribute. Notably, however, in all but one subject for whom a clear OERP was recorded, we also observed a distinct response from the more peripheral external nasal electrodes. An illustration of the distinction between recordings obtained from these two sources is shown in Figure 1.

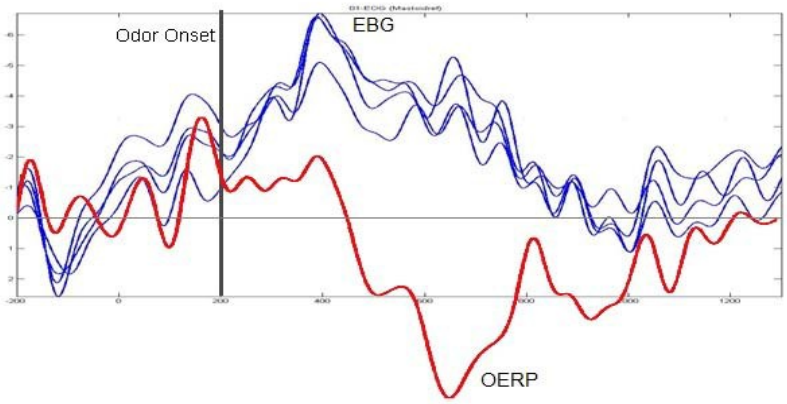


Fig. 1 OERP (RED) at Cz electrode superimposed on responses from the 4 external nasal electrodes (BLUE)

This is a complex data set, which we are still analyzing. Preliminary findings include the following significant observations:

Both threshold and OERP findings indicate changes in olfactory function with intermittent exposure. Our initial OERP analyses indicate that N1 responses (early negative—likely reflecting primarily a more peripheral, sensory response) change with repeated exposure, as do late positive responses (LPR, likely reflecting higher level, perceptual changes), but the latter are only impacted in subjects who received CE training, not in those in the ME group. This is a novel observation that points to the additional impact on olfactory perception that cognitive engagement may bring to intermittent odor exposures. See Figure 2. These findings will be presented in poster form at the 2013 meeting of the Association for Chemoreception Sciences.

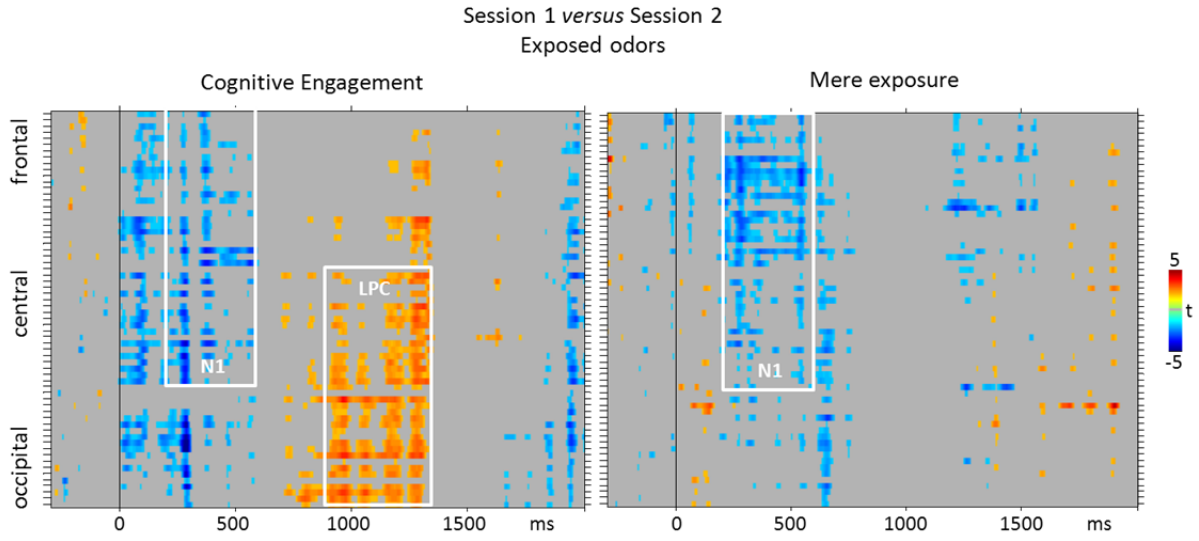


Fig. 2: **Session effects for the two exposure conditions.** Left: CE training was associated with changes in ERP amplitudes during the N1 and LPC periods. Right: Mere exposure was associated with altered ERP amplitudes during the N1 period only.

Discussion

Our initial observations are consistent with the idea that intermittent exposure to complex, natural odors can enhance sensitivity to those odors even in relatively young, healthy individuals who do not evidence prior insensitivity to the odors. Moreover, if the exposure occurs within the context of a task that cognitively engages the subject, further perceptual enhancement may occur.

We have also demonstrated that external nasal electrodes yield a consistent response to odor stimulation that is distinct from the central response recorded by traditional ERP electrodes. This will enable us to further distinguish peripheral and central mechanisms underlying changes in sensitivity, and to determine whether the context of exposure affects their relative contributions. It may also provide a tool for the objective physiological measurement of olfactory sensitivity in clinical settings.

Project 7: Mechanisms of rapid olfactory learning

Johan Lundström

Executive summary

The general goal of this subproject was to determine the central mechanism of rapid olfactory learning and its impact on olfactory processing by means of four interconnected experiments using both behavioral and functional neuroimaging methods. Our data demonstrates that pairing an odor with a brief aversive stimulus increases the individual's absolute detection sensitivity with an average of 20% compared to a control odor. These fast and plastic responses were mediated by interactions between processing in early olfactory neural areas and areas processing memories. This suggests that the conditioning-dependent increase in odor sensitivity is mediated by interplay between sensory and associative mechanism. Finally, we can demonstrate that the plasticity of the individual's sensory acuity is caused by transient mechanisms that dissipate a few days after the aversive pairing unless the negative associations are maintained. Taken together, the results from this subproject clearly demonstrate that aversive conditioning selectively increases an individual's ability to detect biologically relevant stimuli as well as augments discrimination in a decision-making task. This implies that aversive conditioning is not only augmenting attentional filters to highlight relevant stimuli in our surroundings but also works to increase sensory acuity in a stimulus specific manner.

Background and Objectives

Aversive conditioning has been demonstrated to cause an organism to attend more towards the conditioned stimuli and also to process the conditioned stimuli in a faster and more direct manner[47]. Among the senses, it appears that olfactory stimuli produce one of the more powerful conditioning responses where only a limited number of pairings is required between the odor and the aversive stimulus for a conditioning response to occur. A likely mechanism behind this phenomenon is the close link between the early stages of the olfactory system, the amygdala, and the hippocampus - subcortical areas responsible for threat detection and memory formation[48]. Recent data suggest that rapid olfactory learning not only produces an aversive response but also increases olfactory processing abilities[49]. This rapid odor learning could be attributed to either one of two mechanisms: *sensory learning* taking place in early olfactory areas (namely olfactory bulb, olfactory tract, or piriform cortex) or *association learning* mediated by attentional and/or emotional aspects, indicated by activity within the amygdala or the hippocampus.

The general goal of this project was to determine the central mechanism of rapid olfactory learning and its impact on olfactory processing. The specific aims of this project was: **1**) to establish the behavioral phenomena of rapid olfactory learning, **2**) to determine potential behavioral long-term effects, **3**) to determine the underlying neuronal mechanism, and **4**) to determine differences and similarities between rapid- and long-term olfactory learning. Five experiments were proposed to address these aims.

General approach

Participants underwent a classical conditioning paradigm where an odor (CS+) is paired with an aversive stimulus (electric shock, US) a total of 9 times per acquisition session. During the behavioral experiments (Study 1, 2, 3, & 5, below) odor detection threshold (main dependent measure) and skin conductance responses (secondary dependent measure)

were assessed before and after aversive acquisition; the latter measure is used to demonstrate successful acquisition. During the neuroimaging experiment (Study 4), cerebral responses to conditioned and non-conditioned odors stimuli were assessed before and after acquisition.

Main Results

Specific Aim 1: to establish the behavioral phenomena of rapid olfactory learning

In **Study 1**, we initially replicated the foundation for the proposal by demonstrating that aversive conditioning increased participants' ability to discriminate between the odors of two previously-indistinguishable enantiomers. This demonstrates that aversive conditioning induces a rapid plasticity within the olfactory system that dramatically alters our olfactory percept (Figure 1a). In **Study 2**, we could demonstrate that the likely mechanism is an altered absolute sensitivity towards the conditioned stimuli. We demonstrated that aversive conditioning of an odor dramatically increased participants' odor sensitivity to the specific odorant (20% increase). The sensitivity towards a target odor was increased post-conditioning if that target odor was paired with shock during conditioning; however, if a control odor was paired with shock during conditioning, the sensitivity towards the target odor decreased, thereby demonstrating the odor specificity of this effect (Figure 1b).

Specific Aim 2: to determine potential behavioral long-term effects

To assess whether the increase in sensory sensitivity was mediated by a permanent or plastic response, in **Study 3**, we assessed detection threshold in individuals participated in Study 2 eight weeks post-conditioning. The results from Study 3 demonstrated that the odor detection threshold returned to baseline levels eight weeks following conditioning (Figure 1b), thus demonstrating the transient nature that the short conditioning paradigm induced (note: only 8 pairings between the odor in question and the aversive stimuli were delivered).

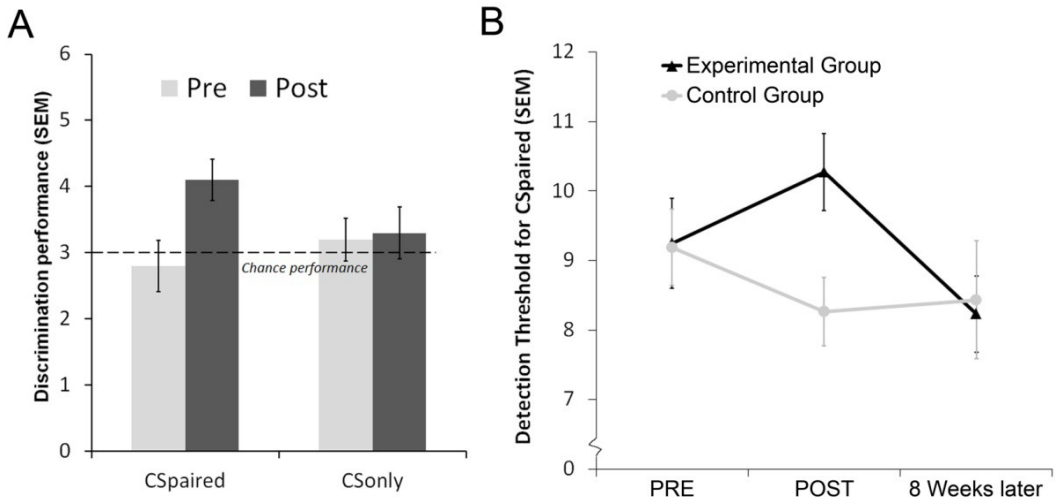


Figure 1.
A) Mean discrimination performance separated by pre- and post-conditioning and odor (CSpaired is the conditioning stimuli). Only performance for the CSpaired in the post-conditioning phase was significantly different from the expected chance value according to a Student's t-test ($p < .001$).
B) Mean odor detection threshold for CSpaired odor in each experimental phase measured in dilution steps. Note that higher values indicate greater dilution of the target odor, i.e., greater sensitivity.

Specific Aim 3: to determine the underlying neuronal mechanism

In **Study 4**, we assessed the neural mechanism of this rapid olfactory learning by means of functional magnetic resonance imaging (fMRI). The comparison of neural processing before odor conditioning to that after odor conditioning demonstrated significant increase mainly in two areas, the hippocampus (Figure 2a) and the olfactory tract (Figure 2b). The olfactory tract is one of the early stages in olfactory processing where odor information start to merge with information relayed via other sensory systems whereas the hippocampus is responsible for memory formation and memory retrieval. This suggests that the conditioning-dependent increase in odor sensitivity is mediated by interplay between sensory (olfactory tract) and associative mechanism (hippocampus).

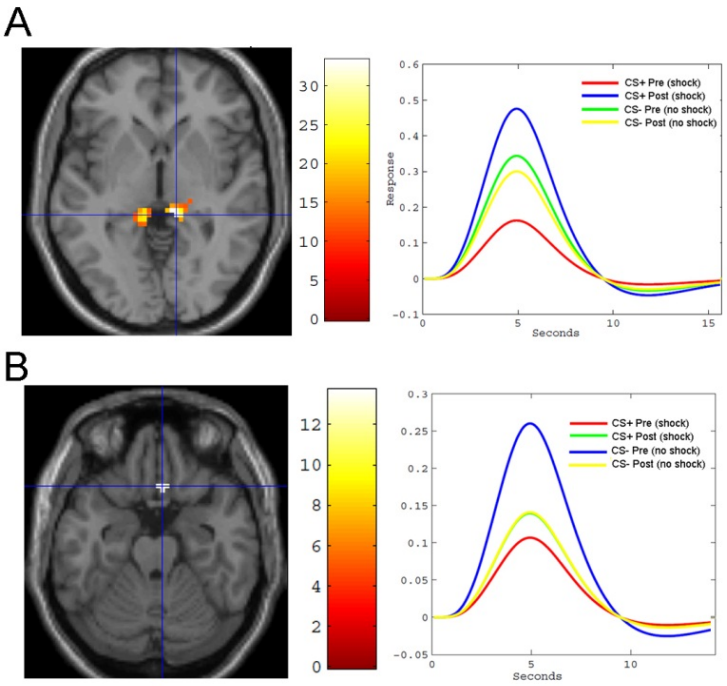


Figure 2. Result from a mixed-model random effect analysis of the interaction between condition phase (Pre and Post conditioning) and odor stimulus (CS+ and CS-) in 26 individuals. Both images restricted by whole-brain FWE correction and percentage signal change extracted from significant voxels within activated cluster marked with crosshair.

A) Significant activity within the bilateral hippocampus in left image with accompanying percent signal change in right image.

B) Significant activation within the right olfactory tract with accompanying percent signal change in right image. Note that left olfactory tract demonstrated a clear, but sub-significant, activity.

Specific Aim 4: to determine differences and similarities between rapid- and long-term olfactory learning.

In **Study 5**, we assessed whether prolonged and repeated pairings (conditioning occurring twice a week for two weeks) between the aversive stimulus and the odor would induce an even greater increased sensitivity or whether the effect is independent of length of learning. In addition, inherent in this question was that we would replicate the results obtained in Study 2, that aversive conditioning increases absolute sensitivity, using another odor. Finally, we aimed to determine whether prolonged pairing with the aversive stimulus would increase the conditioned response to the odor or whether the system would demonstrate extinction to the still active stimulus. As in Study 2, aversive conditioning made participants more sensitive to the paired odor even though extended over a two week period (Figure 3A). This constitutes a replication of the results obtained in Study 2. Moreover, participants exposed to prolonged aversive conditioning demonstrated a decreased arousal response over time as demonstrated by the skin conductance measures (Figure 3B) to the conditioned stimulus. These data, for the first time, provide a demonstration of the biological underpinnings of cognitive behavioral therapy (CBT). Finally, there was no significant

difference in effect-size between results obtained in Study 2 and Study 5. In other words, the demonstrated increase in sensitivity to the conditioned stimulus was independent of the length of exposure under these experimental conditions. No apparent differences seem to exist between rapid and long-term olfactory learning.

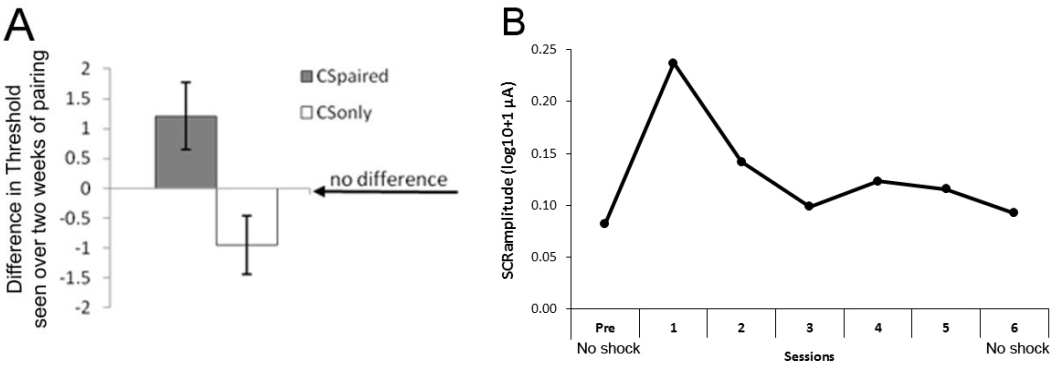


Figure 3 A) Aversive conditioning increased sensitivity towards the conditioned odor (CSpaired; $p < .02$) after two weeks of repeated exposure (tested two to three days after last pairing). **B)** As demonstrated by the conditioned response (skin conductance response) to the CS+, responses decline over time even in the presence of the unconditioned stimulus (UC), the shock. This constitutes the first experimental demonstration of a decrease in conditioned response after prolonged exposure to the UC, the theoretical base for the cognitive behavioral therapy repeated exposure technique.

Significance and implications

The results from these studies clearly demonstrate that aversive conditioning selectively increases an individual's ability to detect biologically relevant stimuli as well as augments discrimination in a decision-making task. This implies that aversive conditioning is not only augmenting attentional filters to highlight relevant stimuli in our surroundings but also works to increase sensory acuity in a stimulus specific manner. Results from Study 4 indicate that this is mediated by interplay between processing in early olfactory sensory areas and higher order association cortex. Moreover, our data from Study 5 clearly demonstrate that the sensory acuity effect is transient in that it undergoes automatic extinction unless the negative associations are maintained. Finally, the extinction over time of the conditioned response (SCR data in Figure 3B), even in the presence of a maintained SCR response to the US as well as the increase in sensory acuity, demonstrates that the increase in sensory acuity is not regulated by the conditioned response per se. However, more importantly, this constitutes the first biological demonstration of the base for cognitive behavioral therapy – extinction of the conditioned response through repeated exposure.

Project 8: Analytical and sensory identification of stress or evasive odors from human subjects

George Preti and Pamela Dalton

Executive summary

Based on evidence from studies using experimental animals which demonstrate that stressed individuals produce a distinctive odor and that these stress odors can be detected by conspecifics, we hypothesized that a distinct, distinguishing odor would arise in stressed humans that could be both recognized by other humans and identified using analytical instrumentation. This stress odor was sought in the axillae because it has been well documented that apocrine glands (which contain axillary odor precursors) and eccrine glands are activated by increases in stress-related hormones. We further reasoned that stress would create a distinct axillary odor with a "sulfurous/onion-like note" due to larger than normal amounts of apocrine secretions arriving on the skin surface being metabolized. Subject recruitment and sweat collection was delayed for almost a year because of IRB-related issues. Following approval we began stress induction of human subjects using the Trier Social Stress Test (TSST). We found that both physiological data (heart rate and electrodermal activity) as well as subjects' subjective ratings demonstrated that subjects were `significantly stressed by the TSST protocol. Using qualitative gas chromatography-olfactometry as well as quantitative objective sensory tests, we also found that, as predicted, axillary secretions collected on pads during stress smell significantly different from pads collected at other times (non-stress/neutral times). Unfortunately, due to delays in obtaining human subject approval we were unable to complete the analytical portion of this study. We retain the stimuli and hope to evaluate these in the future.

Background and Objectives

There is considerable evidence from studies on experimental animals as well as several studies using humans which demonstrate that stressed individuals produce a distinctive odor. These stress odors can be detected by conspecifics and can affect their behavior and physiology. Odors associated with stress are thought to arise because of an increased production of body odorants mediated by increases in stress-related hormones. However, the chemical identity of these odorants has not been determined.

The use of odor as a biometric for stress or evasive behavior has major advantages because it is non-invasive, represents minimal risk to personnel collecting the samples, and can be sampled frequently and remotely if desired. In addition, detection of this odor by other humans may result in physiological changes in the recipient, as has been documented in non-human mammals and suggested by our preliminary results. Consequently, the two aims of this research were the following:

(**1)** To identify the type and abundance of volatile odorants associated with stress in humans and

(2) To determine their temporal and quantitative relationships to other stress-related biomarkers such as heightened cortisol levels, heart rates and respiration rates.

Potential benefits to deployed personnel and warfighters for our research include (a) real-time, noninvasive individual monitoring of an individual's physiological status and health and (b) detection of stressed or deceptive individuals at restricted entry points.

Progress towards objectives: After a delay of almost a year, we obtained IRB-approval from both the University of Pennsylvania and Army IRBs for all of our experimental protocols, including stress induction in human subjects via the Trier Social Stress Test (TSST). The TSST was chosen because it has been shown to have a profound effect on salivary cortisol and other measures of autonomic reactivity, such as heart rate and skin conductivity.

Protocol: Briefly, all subjects go through a 10 day wash-out period using unfragranced liquid soap and come to the laboratory for two sessions. On day one data and samples (axillary sweat pads; axillary extracts; saliva; heart rate, self-stress ratings) are collected without subjects being stressed **(Neutral Day).** On day two these same data and samples are collected under stress (**Stress Day).**

Subject Accrual: We have collected data from 18 individuals: thirteen males: (3 Caucasians, 8 African Americans and 2 Asians) and five females (4 Caucasians, 1 African American). From each subject we have available two neutral day extracts (one from each axillae), two stress day extracts as well as two sets of axillary pads from the neutral day (4 pads/subject) and from the stress day (4 pads/subject).

Results

Physiological data: The data gathered from subjects participating in our protocol demonstrate that they were stressed by the TSST procedure as indicated by both the heart rate increase (see Figure 1) and skin conductance measures (see Figure 2). Both of these parameters show significant increases brought-on by the stress day procedure. Salivary cortisol and α-amylase are still being processed.

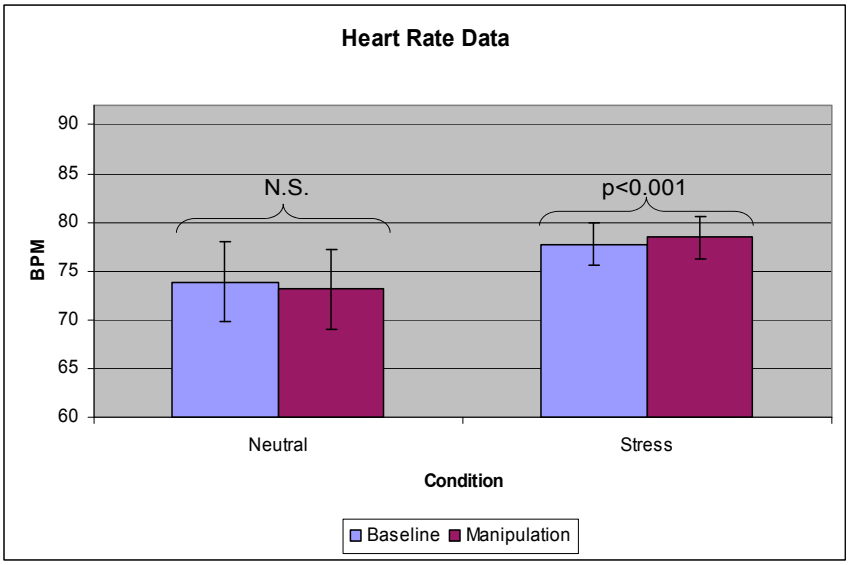


Figure 1. Using an Analysis of Variance (ANOVA) for each Condition, we see no statistical significance in the decrease in Heart Rate from Baseline to Manipulation during the Neutral Condition, $F(1,17)=0.33209$, $p=0.57199$, whereas Heart Rate increased significantly from Baseline to Manipulation during the Stress Condition, $F(1,17)+16.022$, $p=0.00092$.

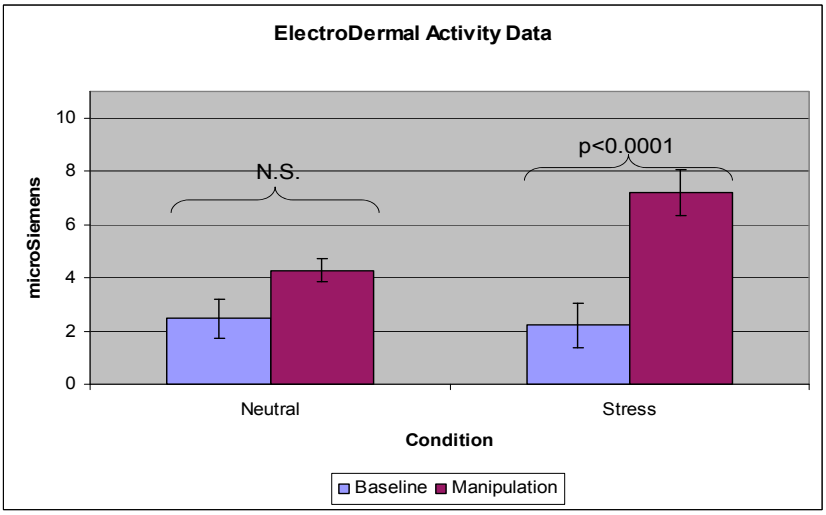


Figure 2. Using a Repeated Measures Analysis of Variance (ANOVA) with Condition (Neutral x Stress) and Time (Baseline x Manipulation) as the Within Effects, a Condition x Time Interaction was revealed, ($F(1,17)=9.8649$, $p=0.00596$) such that, when a Bonferroni Post Hoc was applied, it was shown that the increase in ElectroDermal Activity from Baseline to Manipulation was significant for the Stress condition ($p=0.000013$) but not the Neutral Condition ($p=0.125449$).

Subjective Ratings: The subjective ratings made by our participants about their mood states throughout the procedure on both the Neutral and Stress Days demonstrate that they were significantly more anxious on the Stress Days as indicated by: Visual Analog Scale (VAS) measures of anxiety (See Figure 3), Profile of Mood State (POMS) Scores of anxiety (See Figure 4), and Derogatis Affective Balance Scale (DABS) Scores of Anxiety (See Figure 5).

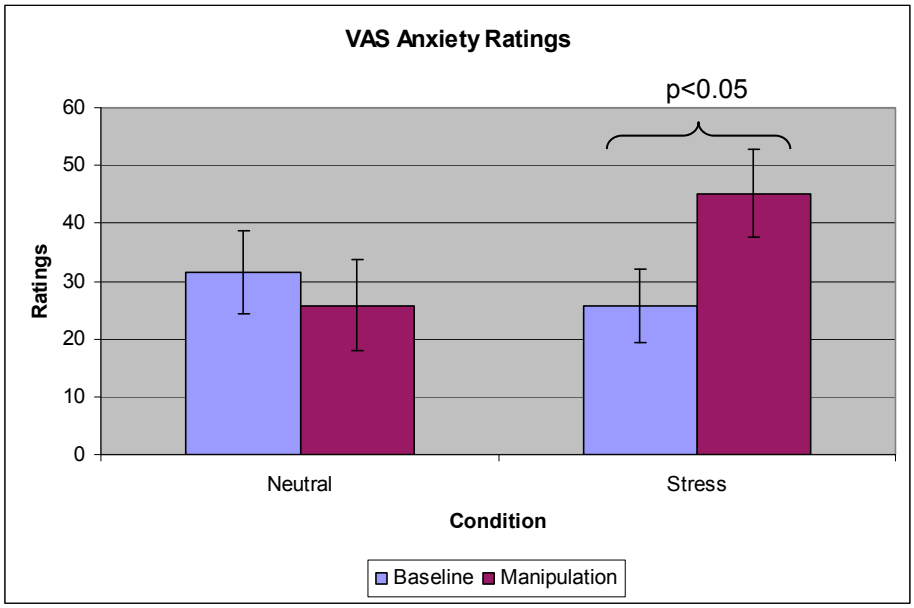


Figure 3. Using an Analysis of Variance (ANOVA) for each Condition, it was revealed that the decrease in Anxiety Ratings using the Visual Analog Scale (VAS) during the Neutral Condition were not statistically significant (F(1,17)=1.08534, p=0.31210), whereas the increase in VAS Anxiety Ratings during the Stress Condition was significant (F(1,17)=5.9737, p=0.02572)

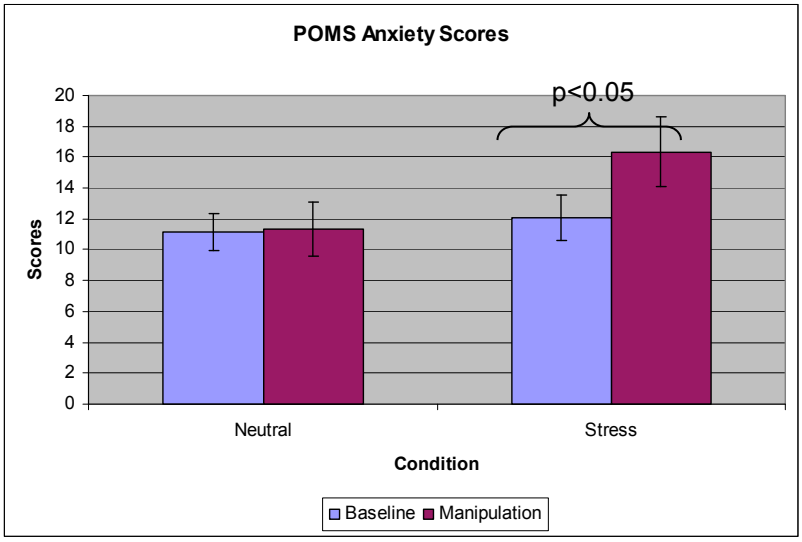


Figure 4. Using an Analysis of Variance (ANOVA) for each Condition, it was revealed that the increase in Anxiety Scores using the Profile of Mood State Scale (POMS) during the Neutral Condition were not statistically significant (F(1,17)=0.04182, p=0.84038), whereas the increase in POMS Anxiety Scores during the Stress Condition was significant (F(1,17)=6.4858, p=0.02085)

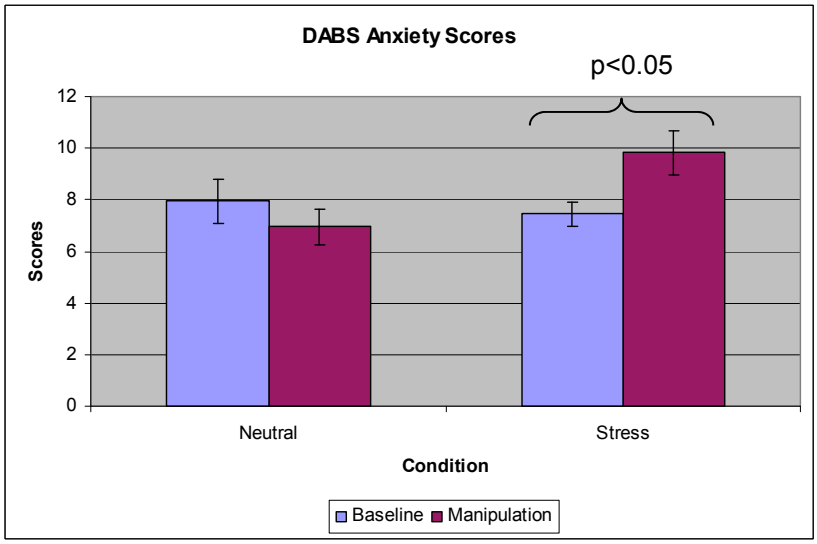


Figure 5. Using an Analysis of Variance (ANOVA) for each Condition, it was revealed that the decrease in Anxiety Scores using the Derogatis Affect Balance Scale (DABS) during the Neutral Condition were not statistically significant (F(1,17)=3.2963, p=0.08712), whereas the increase in DABS Anxiety Scores during the Stress Condition was significant (F(1,17)=8.3513, p=0.01018)

Gas-Chromatography-Olfactometry (GC/O) qualitative evaluation of extracts: Pooled samples of Neutral Day extracts and pooled samples of Stress Day extracts were made by combining 50µl of each sample. These were initially used for both informal sensory evaluations as well as GC/O analysis.

Informal sensory evaluation of the pooled extracts by both the Dalton and Preti lab members suggest a difference in odor quality noticeable above the ethanol solvent between stress and neutral day extracts, with the former having more axillary-odor quality than the latter.

GC/O is a qualitative, descriptive technique to help identify what types of odorants are present in the sample and where they elute in the chromatogram. Each pooled sample was independently evaluated by 5 judges and their ratings examined for body odor (e.g., sweaty, sour, B.O., acidic) or oniony-sulfurous responses. The latter is important to our hypothesis that sulfurous odor notes are part of the stress odor signature.

Table 1. Total number of odors eluting with body odor or sulfur/onion-like quality.

These are summaries of perceived body odor notes from all judges in the GC/O experiments. Body odor descriptors included sour, acidic, "b.o.," armpits, axillary odor, sulfurous, axillary thiol, "onion b.o." The number of onion-like notes is shown in parentheses. Our hypothesis is that sulfur compounds with onion-like qualities characterize stress odor.

Sample	Responses
Neutral Day	27 "body odor" responses from all judges; acidic, sour, onion-like (7).
Stress Day	35 "body odor" responses from all judges; acidic, sour, onion-like notes in greater abundance (13).

We performed a log linear analysis based on count data and found that the main effect of stress was not significant but there was a significant difference in the body odor responses vs. onion odor responses. The Chi sq was 5.458, $p < 0.019$. We believe that the body odor responses would be significant across the neutral and stress conditions but the number of odor judges would have to be expanded to gain more power: e.g., 30 subjects to observe significant stress-neutral differences at a power of 0.80.

GC/MS data: Small aliquots analyzed independently, by 5 judges (3 males; 2 females) using GC/O.

Despite the fact that there is a perceptual difference in the extracts from neutral and stress days, GC/MS data are unremarkable in that the un-concentrated extracts [5µl injected from

the pooled axillary extracts from the neutral and stress day] show no clearly obvious differences: **see Figure 5a**. The large components at approximately 33 minutes, and beyond are non-odorous skin lipids.

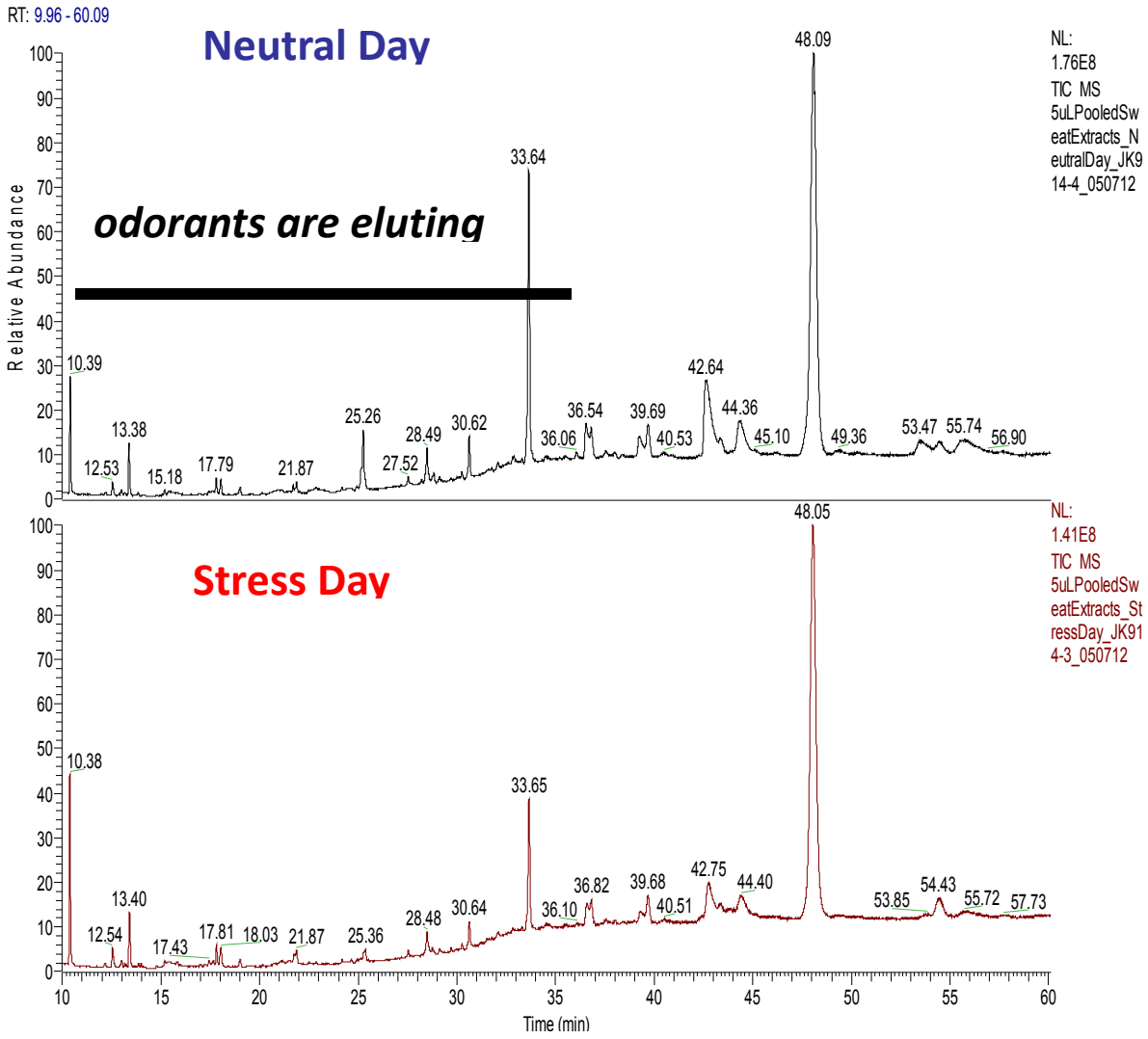


Figure 5a. Total Ion Chromatograms from pooled axillary extracts from both neutral day [top] and stress day [bottom]. Solid bar in the top chromatogram shows the time interval where all odorants elute (as determined from the GC/O experiments).

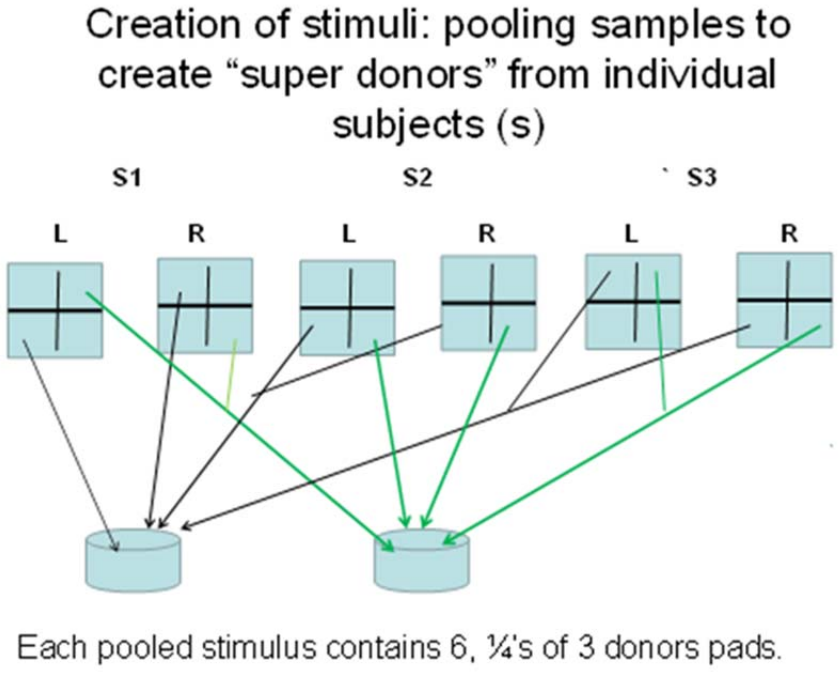
We have submitted the GC/MS data from the un-concentrated extracts for full metabolomics analyses using the MeDDL software package developed by collaborators at the Air Force Laboratory, however, due to the size of the data files and their other commitments, we are waiting.

Axillary Pads:

We have examined the odor differences between axillary pads collected during the neutral day and stress day and find significant sensory differences. The procedures employed to examine sensory differences between axillary pads worn during the neutral day procedure and the stress day procedures were as follows

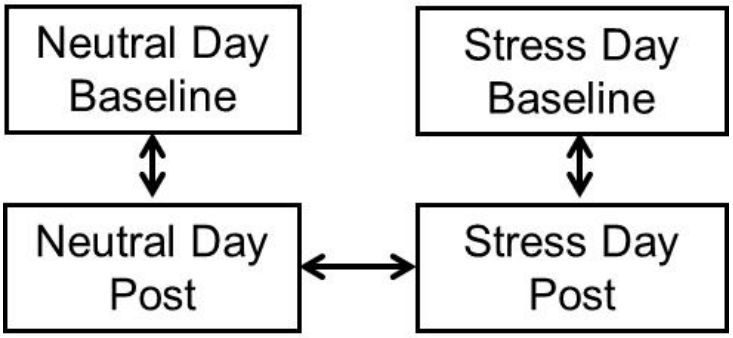
Each subject donated 2 axillary pads (1 from each axillae) for each of 4 conditions; hence for each subject there are 2 pads from Neutral Day Baseline, 2 pads from Neutral Day Video, 2 pads from the Stress Day Baseline and 2 pads from the Stress Day Induction. We hypothesized that the pads from the stress induction period would be most distinguishable and different from all other pads.

The pads were stored frozen @ -80C until processing for sensory testing and analytical studies. For sensory testing we created pooled samples from pads. Each pad was cut into 4 pieces to help create "super donors" from individual subjects (S). Two of the pieces were employed for sensory stimuli and two were re-frozen for analytical studies. Each pooled stimulus sampled contained 6, ¼'s of 3 donors' pads, as shown in the diagram below.



Each pooled stimulus contains 6, ¼'s of 3 donors pads.

A triangle testing design was used to determine if pooled pad samples from neutral or stress days differed significantly from one another. We employed 6 female evaluators, aged 21-41; pre-screened for olfactory sensitivity. They evaluated all samples in a randomized order; each of 3 comparisons tested 6 times (n=18 trials/session) as shown here:



To determine whether or not the evaluators were able to select the stress sample compared to the neutral sample at above chance levels, the data were normalized such that the scores were converted to proportion correct and compared to the 33% random chance that an evaluator would select a given sample. Using an ANOVA, it was shown that the proportion correct for the Stress vs Neutral condition was significantly higher than the Neutral vs. Neutral Baseline Condition ($F(2,10)=6.4190$, $p=0.01610$. Using a Chi Square Analysis, with Proportion Correct used as the Observed Frequency and 0.33 as the Expected Frequency, it was revealed that the Chi Square = 20.50639, $p=0.000035$, showing that these ratings are significantly above chance. The results of this analysis are shown in **Figure 6**.

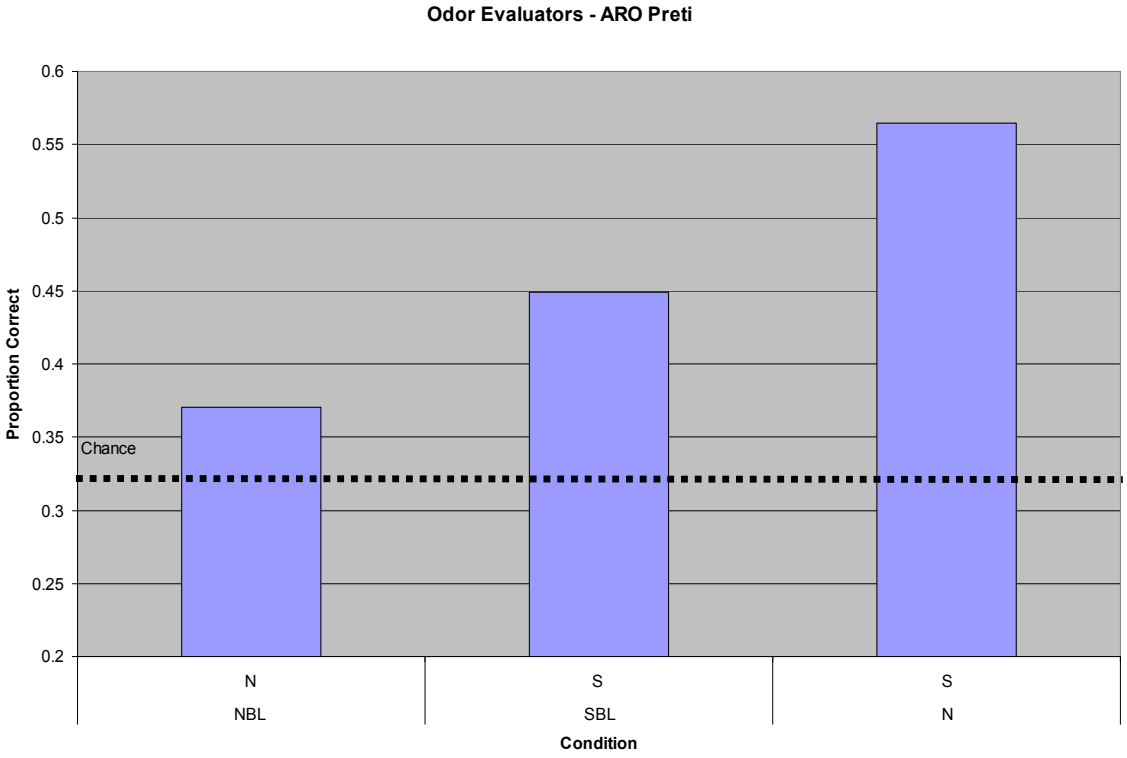


Figure 6. Using a Chi Square Analysis, with Proportion Correct used as the Observed Frequency and 0.33 as the Expected Frequency, it was revealed that the Chi Square = 20.50639, $p=0.000035$, showing that these ratings are significantly above chance.

Discussion and Future Plans:

The analyses performed to date demonstrate that subjects are `effectively stressed by the TSST protocol and that the odor of axillary secretions collected on pads during stress smell significantly different from pads collected at other times (non-stress/neutral times). Consequently, our sensory data demonstrate that our hypothesis is correct: axillary odor is altered by stress-related situations.

Unfortunately, the delay and confusion surrounding the IRB approval for this study has delayed more in-depth analytical elucidation of the volatile compounds responsible for sensory differences. This is still being examined using both the axillary extracts and pooled samples of pads. For analytical studies involving organic solvents to extract volatile compounds from the pads, we are pooling the pads into identical “super donors” we created for the sensory testing.

The qualitative GC/O studies we have done suggest that differences are present between the stress and non-stress samples, but these are likely to be quantitative differences in certain compounds with sulfurous and body odor olfactory qualities: e.g., organic acids such as 3-methyl-2-hexenoic acid sulfur-containing compounds such as 3-sulfanyl-alcohols These and other compounds which the metabolomics analyses may find to be differentially influenced by stress are the target of further analytical studies as we wish to document their structures.

Project 9: Effects of stress on odorant memory accuracy and duration

Charles Wysocki

Executive Summary

The aim of these studies was to determine the effects of stress on odor memory. The influences of two forms of stress were to be evaluated: physiological and combined physiological and psychological[50,51]. Study one utilized the former; study two was designed to expand on this by incorporating psychological stress.

Both studies were successful at inducing stress (see details below). In the first study there appeared to be a gender-specific interaction between stress and odor memory recall; women appeared to be more affected by stress than men. This was not replicated in the second study. Context during learning and memory recall also was evaluated and proved not to matter. Neither study could make a strong case for an influence of stress during odorant exposures on subsequent odor recall.

Background and objectives

Odors can play a significant role in memory retrieval. Subjects who learned a specific task in the presence of vanilla odor and were later tested in the same odor environment outperformed those who learned in the odor environment and were tested in a different context[52]. Importantly, subjects who were stressed during initial learning performed more poorly than those who were not stressed; however, testing in the same odor context that was present during initial learning ameliorated the effects of stress[52]. From case- and post-exposure-studies, odors also appear to have significance in posttraumatic stress [PTS][53] and apparently can elicit panic attacks[54]. More recent work suggests that the influence of odors in PTS can be quite long-lasting[55]. Although odors elicit more emotional memories than other stimuli[1], no experimental studies on the apparent long-term influence of stress on odor memory per se have been published. On the one hand, if stress disrupts learning in general then this also should apply to odor learning and memory; however, the case reports and other studies of PTS suggest that odors experienced under stress may be more engrained and long-lasting.

STUDY 1

Methods: Physiological stress was induced via the cold pressor test (submersion of a hand in ice-cold water). The manipulation successfully induced stress (Fig. 1). During the odor learning phase, after stress was induced five odorants were presented. In the odor recall phase, two weeks later, these five odorants were embedded in a total of 20 odorants. The task of the subject was to determine whether the test odorant had been present during the initial session.

A total of 64 individuals completed the experiment (32 women): Asian, 8; Black (not Hispanic), 8; Caucasian, 35; Hispanic, 6; Unknown/Other, 7. Their ages ranged from 21 to 46 years (average = 27 ± 5 SD). They were assigned to one of four conditions in a 2 X 2 design: stress during both odor learning and odor memory recall; no stress during both phases; or stress only during one or the other of the two phases (two conditions). Extant literature would predict that 1) stress during initial odor exposure should interfere with learning (although the clinical literature related to PTS suggests that odor stimuli may be an exception since odor per se may exacerbate episodes of PTS); 2) odor presence

during both learning and memory recall should ameliorate the effects of stress; 3) environmental consistency during learning and recall should facilitate recall, irrespective of stress condition (although the double non-stressed subjects might be expected to outperform all others) and inconsistency in the phases should have a negative impact on learning and recall.

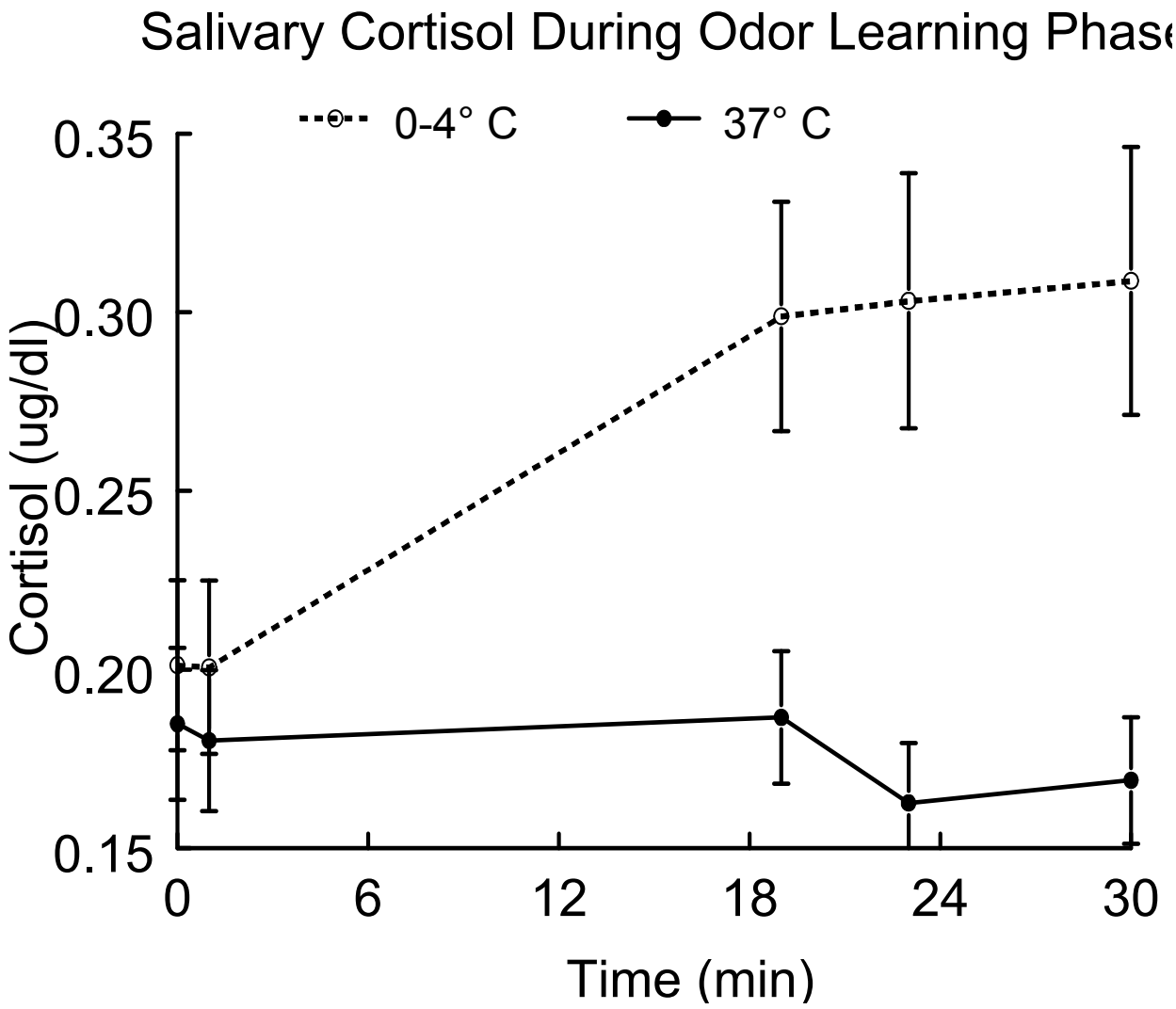


Fig. 1. Elevated salivary cortisol reflects induced stress in individuals who immersed a hand in cold water twice; once, just after time 0, and again at time 20 min (data not shown). BP, but not HR, was elevated (not shown).

Results: Race/ethnicity sample sizes in many groups were too small to evaluate potential differences. When entered as a covariate, age did not play a significant role in any of the analyses of variance.

During odor learning, not surprisingly, gender influenced blood pressure: men had higher readings than did women. Blood pressures were elevated in both males and females during exposure to cold. Self-reports of pain and unpleasantness were also significantly elevated during exposure to cold and they were significantly greater in women exposed to cold than

in men ($p < 0.06$ for each interaction). These gender differences were either absent or less pronounced during the odor memory recall phase.

Analysis of variance of the total correctly recalled odors during the memory recall phase revealed that there was a significant interaction between gender and stress condition during the learning phase (Fig. 2). Women were better able to recall odors experienced during stressful odor exposure than were men, but this was reversed when the initial odors were experienced in the absence of the cold pressor exposure. Restricting analysis only to those who experienced cold stress during learning revealed that irrespective of the water bath temperature during the recall phase, women tended to outperform men ($p = 0.07$; Fig. 3).

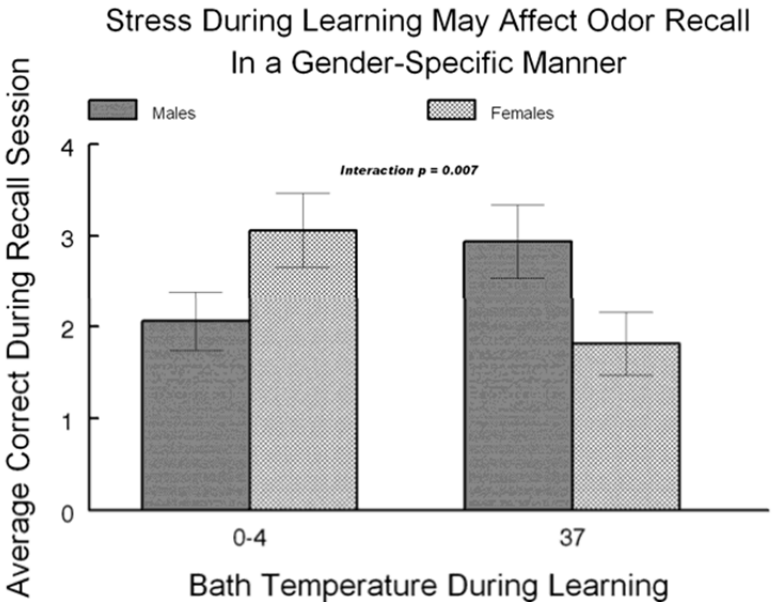


Fig. 2. Odor memory recall was influenced by both gender and stressor condition during the odor learning phase of the experiment. When stressed during learning, women later outperformed men recalling more odors correctly. This gender difference was, however, reversed when stress was absent during learning.

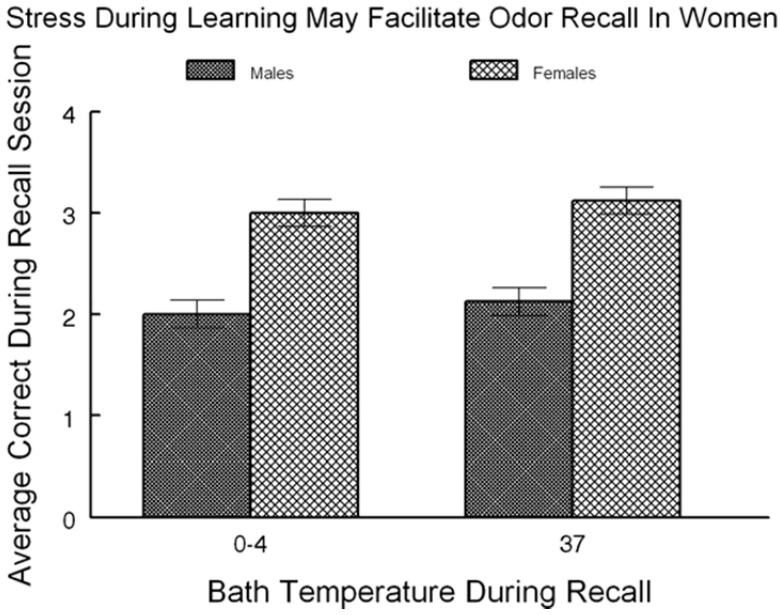


Fig. 3. Exposure to odor during stress in women tended to facilitate correct odor recall in a later memory test. This was not found in the unstressed learning phase of the experiment.

Discussion: Exposure to odors during stress had a greater effect in women than in men, which is consistent with general findings in the olfactory literature, viz., women tend to "outperform" men in measures of olfactory performance. Why this occurred only in the learning/stress phase in women is unknown. As used above, "outperform" should not be interpreted as showing a positive response to the odors. Women may have been more negatively affected by stress during odor learning than were men, which may have contributed to the outcome.

The working hypothesis posited that when the context during recall is the same as when initial learning occurred then recall should be facilitated. In general, the results did not support this hypothesis. Indeed, to the contrary, women who were stressed during learning had the best overall odor recall during the memory test, irrespective of the context during the recall task.

STUDY 2

Introduction: Study two was designed as a replication and extension of study one. The same physiological stress (cold pressor test) was used but volunteers also were exposed to psychological stress. A webcam was placed in front of each participant who was informed that another, opposite-sex investigator, in a separate room, would be evaluating performance during the experiment (this was not true and as such, at the end of the subject's participation, each was debriefed and informed of the deceit).

Methods: A total of 66 volunteers were recruited for participation, but 2 failed to pass an odor screening test; there remained 32 of each gender. There were 12 Asian/Pacific Islanders, 10 Blacks (non-Hispanic), 2 Hispanics, 24 Caucasians, and 6 Unknown/Others. In analyses, race was not included as a factor because of the relatively small sample sizes in some groups. Ages ranged from 21-50 years with an average of 27.8 (±8). In the analyses that follow, age was a significant covariate only for measures of blood pressure.

Four groups were formed depending upon which water bath was experienced in the learning phase (warm or cold) and in the odor recall phase (warm or cold). Half of the participants experiencing warm in the first phase also experienced warm in the second phase; the same was true for those experiencing cold: These individuals formed groups in which the odor learning and odor memory phases were internally consistent, i.e., the context was the same. The remaining participants experienced both temperature conditions, although in different phases (balanced for which bath temperature occurred first): These individuals formed groups in which the context of the two phases differed. Within each of the four groups there were 8 women and 8 men.

Within a session baseline physiological and subjective measures were obtained. A hand was then inserted into the water bath for at least one minute but preferably for three. Measures were repeated at minutes 1 and 19. The hand was reinserted into the bath for at least one minute (up to three). Odorants were then presented (five during the learning phase and 20 during recall). Measures were repeated a final time upon completion of the odorant exposures.

Results: As in the first study, the cold pressor test was successful at inducing stress during the first phase (odor learning); volunteers exposed to the cold water had significantly elevated cortisol ($F_{(4, 248)} = 9.98$, $p < .001$; there was no effect of gender). Unexpectedly, during the second phase (odor recall) there was no significant effect of water bath temperature on salivary cortisol levels; however, other physiological and psychological (see below) measures support the conclusion that stress was indeed present in the second phase. In both phases of the experiment both age and gender typically has significant influences on blood pressure (both systolic and diastolic), but this is a general finding in the biomedical literature. Importantly, there were significant interactions between blood pressure readings and experimental group assignment (all $p < .05$ but typically $<.0001$); during exposures to cold, in both phases, both systolic and diastolic readings were elevated. Heart rate declined over the course of each phase ($p < .001$) and there was a tendency for an interaction between gender and water bath temperature; males tended to

have higher rates during exposures to cold and females had higher rates during exposures to warm ($p < .04$ in the first phase and $p < .07$ in the second).

Self-ratings of pain stress and pleasantness of the situation tended to agree with the physiological measures. Pain was elevated during exposures to cold in both phases ($F's_{(1,60)} = 26.35$ & 17.71, respectively, $p < .0001$). Importantly, in each phase there were significantly elevated reports of pain during exposures to cold (interaction $F's_{(4, 240)} = 24.83$ & 31.56, respectively, $p < .0001$). Very similar, significant ($p < .0001$) interaction patterns were noted for self-reported stress (which were elevated during exposures to cold) and for ratings of pleasantness/unpleasantness of the situation; during exposures to cold there were elevated ratings of unpleasant.

Physiological measures and psychological reports are meaningful just as subjects enter the odor-exposure component of the first phase of the experiment. Blood pressure measures were significantly elevated ($p < .05$) in individuals exposed to cold water, relative to these measures in individuals exposed to warm water, but cortisol was not. All of the psychological reports also were elevated in these individuals ($p < .0001$). These data suggest that individuals were stressed as they were exposed to odors; however, all of these differences were absent at 30 minutes (the end of the learning phase). The same pattern of stress was noted just prior to exposure to odorants during the odor memory recall phase; however, all significant differences were absent at 35 minutes (end of the recall phase).

Women tended to have better odor recall than did men (3.16 versus 2.56; $p < .09$). There were neither significant effects of group assignment ($F_{(3,64)} = 0.6$, $p = .62$) or context consistency ($t_{(62)} = 1.19$, $p = .24$) nor significant interactions among factors on odor memory recall.

Odor memory recall in participants who were physiologically stressed in the first session did not differ from those who were not physiologically stressed ($t_{(60)} = .09$, $p = .93$).

Discussion: Although, as in study one, the cold pressor test to induce stress appeared to be successful the effects of psychological stress cannot be properly evaluated. In design the psychological stress was to have been combined with the physiological stress. Although this did occur, participants in the warm water sessions also were provided with the misinformation about a second experimenter. This was an error in implementation that was not detected until completion of the study. This could have had an impact on either odor learning or odor recall or both in the control (warm-warm) group.

The significant interaction between odor memory, gender and water bath temperature during initial exposures to odor, noted in study one, did not replicate in this study.

Consistency of environmental context facilitates visual memory recall and the presence of odor during learning while under stress and its subsequent presence during memory recall appear to ameliorate the effects of stress. This may be true for environmental odor in general, but it does not appear to facilitate odor memory recall per se when numerous odorants are sequentially presented during stress.

Perhaps a modification in experimental design could reveal additional findings. Instead of focusing on odor memory recall, odor can be paired with stress, e.g., throughout phase one, which could also incorporate additional stressful tasks between the two water bath emersions. In a subsequent, shorter session, participants would enter the same room with or without the odor that was present during stress and physiological and subjective data would be recorded. Additional control groups would include: stress, but no odor

subsequently followed by exposure to odor or no odor. The same four groups could be formed but there is no stress during phase one. This design could determine whether odor, previously experienced during stress, could itself later elicit symptoms of stress.

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